

APPLICATION
FOR
UNITED STATES LETTERS PATENT

TITLE: NOVEL MOLECULES OF THE CARD-RELATED
PROTEIN FAMILY AND USES THEREOF

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NOVEL MOLECULES OF THE CARD-RELATED PROTEIN
FAMILY AND USES THEREOF

Related Application Information

This application claims priority from provisional application serial number 60/161,822, filed October 27, 1999. The entire content of this application is herein incorporated by reference.

Background of the Invention

In multicellular organisms, homeostasis is maintained by balancing the rate of cell proliferation against the rate of cell death. Cell proliferation is influenced by numerous growth factors and the expression of proto-oncogenes, which typically encourage progression through the cell cycle. In contrast, numerous events, including the expression of tumor suppressor genes, can lead to an arrest of cellular proliferation.

In differentiated cells, a particular type of cell death called apoptosis occurs when an internal suicide program is activated. This program can be initiated by a variety of external signals as well as signals that are generated within the cell in response to, for example, genetic damage. For many years, the magnitude of apoptotic cell death was not appreciated because the dying cells are quickly eliminated by phagocytes, without an inflammatory response.

The mechanisms that mediate apoptosis have been intensively studied. These mechanisms involve the activation of endogenous proteases, loss of mitochondrial function, and structural changes such as disruption of the cytoskeleton, cell shrinkage, membrane blebbing, and nuclear condensation due to degradation of DNA. The various signals that trigger apoptosis are thought to bring about these events by converging on a common cell death pathway that is regulated by the expression of genes that are highly conserved from worms, such as *C. elegans*, to humans. In fact, invertebrate model systems have been invaluable tools in identifying and characterizing the genes that control apoptosis. Through the study of invertebrates and more evolved animals, numerous genes that are associated with cell death have been identified, but the way in which their products interact to execute the apoptotic program is poorly understood.

Caspases, a class of proteins central to the apoptotic program, are responsible for the degradation of cellular proteins that leads to the morphological changes seen in cells

undergoing apoptosis. Caspases (cysteinyl aspartate-specific proteinases) are cysteine proteases having specificity for aspartate at the substrate cleavage site. Generally, caspases are classified as either initiator caspases or effector caspases, both of which are zymogens that are activated by proteolysis that generates an active species. An effector caspase is activated by an initiator caspase which cleaves the effector caspase. Initiator caspases are activated by an autoproteolytic mechanism that is often dependent upon oligomerization directed by association of the caspase with an adapter molecule.

Apoptotic signaling is dependent on protein-protein interactions. At least three different protein-protein interaction domains, the death domain, the death effector domain and the caspase recruitment domain (CARD), have been identified within proteins involved in apoptosis. A fourth protein-protein interaction domain, the death recruiting domain (DRD) was recently identified in murine FLASH (Imai et al. (1999) *Nature* 398:777-85).

Caspases comprise a multi-gene family having at least 12 distinct family members (Nicholson (1999) *Cell Death and Differentiation* 6:1028). A relatively small fraction of cellular polypeptides (less than 200) are thought to serve as targets for cleavage by caspases. Because many of these caspase targets perform key cellular functions, their proteolysis is thought to account for the cellular and morphological events that occur during apoptosis. Members of the caspase gene family can be divided by phylogenetic analysis into two major subfamilies, based upon their relatedness to ICE (interleukin-1 β converting enzyme; caspase-1) and CED-3. Alternate groupings of caspases can be made based upon their substrate specificities.

Many caspases and proteins that interact with caspases possess a CARD domain. Hofmann et al. ((1997) *TIBS* 22:155) and others have postulated that certain apoptotic proteins bind to each other via their CARD domains and that different subtypes of CARD domains may confer binding specificity, regulating the activity of various caspases, for example.

CARD-4 is a member of the CED-4/Apaf-1 family that interacts with RICK, a serine threonine kinase, and induces NF- κ B via the signaling protein TRAF-6 and NIK (Bertin et al. (1999) *J. Biol. Chem.* 274:12955). CARD-4 includes domains that are similar to the nucleotide binding site domain (NBS) and leucine rich repeat (LRR) domains found in plant R proteins that mediate resistance to pathogens.

Summary of the Invention

The invention features nucleic acid molecules encoding human CARD-12. CARD-12 has a CARD domain, a nucleotide binding site (NBS) domain, and a leucine

rich repeat (LRR) domain. These domains are found in a number of proteins that transmit signals that activate apoptotic and inflammatory pathways in response to stress and other stimuli. Upon activation, CARD-12, like Apaf-1 (Zou et al. (1997) Cell 90:405-413), likely binds a nucleotide, allowing CARD-12 to bind to and activate a CARD-containing protein via a CARD-CARD interaction, leading to modulation of apoptosis.

CARD-12 nucleic acids and polypeptides, as well as modulators of CARD-12 activity or expression, are expected to be useful in the modulation of stress-related, apoptotic and inflammatory responses, e.g., for the treatment of apoptotic and inflammatory disorders. In addition, CARD-12 nucleic acids and polypeptides are expected to be useful in the diagnosis of apoptotic and inflammatory disorders as well as in screening assays which can be used to identify compounds which can be used to modulate stress-related, apoptotic and inflammatory responses.

Many cytoplasmic plant proteins involved in response to plant pathogens, generally referred to as "R" proteins, have both an NBS domain and an LRR domain (van der Bizen and Jones (1999) Current Biology 8:226-228). R proteins are involved in both a rapid defense response (hypersensitive response) and more long-term nonspecific resistance (systemic acquired resistance). The hypersensitive response involves cell and tissue death that is localized to the site of infection. The LRR domains of R proteins are believed to recognize and bind to pathogen proteins, triggering defensive responses. Many R proteins have an amino terminal effector domain (e.g., a TIR domain or a leucine zipper domain) that is thought to play a role in downstream signaling of events triggered by infection and, possibly, other stresses.

The R proteins have some structural similarity to APAF-1, a protein which mediates between Bcl-2, a negative regulator of apoptosis, and caspases. A domain, designated the NB-ARC domain ("nucleotide-binding adaptor shared by APAF-1, certain R gene products and CED-4") contains a series of motifs and residues that are conserved among R proteins and APAF-1 (van der Bizen and Jones (1999) Current Biology 8:226-228). In addition to the NBS domain, APAF-1 has a CARD domain, functionally analogous to the effector domain of R proteins, and a WD-40 domain, functionally analogous to the LRR domain of R proteins.

Similar to CARD-12, CARD-4 and CARD-7 have both an NBS domain and an LRR domain as well as a CARD domain (detailed information concerning CARD-4 and CARD-7 can be found in U.S. Application Serial No. 09/245,281, filed February 5, 1999, U.S. Application Serial No. 09/207,359, filed December 8, 1998, U.S. Application Serial No. 09/099,041, filed June 17, 1998, U.S. Application Serial No. 09/019,942, filed

February 6, 1998, and U.S. Application Serial No. 09/428,252, filed October 27, 1999, all of which are incorporated herein by reference). The CARD domain, which is present in a number of apoptotic signaling molecules, is an effector domain that thought to be involved in homophilic protein-protein interactions, e.g., with downstream CARD-containing signaling molecules. For example, the CARD domain of CARD-4 interacts with the CARD domain of RICK (RIP2, CARDIAK), a serine-threonine kinase that activates NF- κ B signaling pathways.

Other proteins structurally related to CARD-12 include NBS-1, Pyrin-1, PCD-1, PCD-2, and PCD-3.

NBS-1 has an NBS domain and a LRR domain, as well as a pyrin domain. Functionally analogous to the CARD domain of CARD-4, CARD-7, and CARD-12, the pyrin domain is an effector domain thought to be involved in homophilic protein-protein interactions. Pyrin-1 also contains a pyrin domain. Detailed information concerning NBS-1 and Pyrin-1 can be found in U.S. Application Serial No. 09/506,067, filed February 17, 2000, which is incorporated herein by reference.

PCD-1, PCD-2, and PCD-3 each contain both an NBS domain and a leucine zipper domain. A leucine zipper domain, like the CARD domain and the pyrin domain, is an effector domain thought to be involved in homophilic protein-protein interactions. PCD-2 and PCD-3 also each contains LRR domains. PCD-1, which is truncated at its carboxy terminus, is also expected to contain an LRR domain. Detailed information concerning PCD-1, PCD-2, and PCD-3 can be found in U.S. Application Serial No. 09/563,876, filed May 3, 2000, which is incorporated herein by reference.

In general, an NBS domain includes a kinase 1a domain (P-loop), and a kinase 2 domain (Walker B box). An LRR domain usually is composed of several leucine rich repeats.

Without being bound by a particular theory, it is possible that CARD-12 participates in the network of interactions that lead to caspase activity. Human CARD-12 may play a functional role in caspase activation similar to that of Apaf-1 (Zou et al., Cell, 90:405-413, 1997). For example, upon activation, CARD-12 might bind a nucleotide, thus allowing CARD-12 to bind and activate a CARD-containing caspase via a CARD-CARD interaction, leading to apoptotic death of the cell.

Accordingly, CARD-12 molecules are useful as modulating agents in regulating a variety of cellular processes including cell growth and cell death. In one aspect, this invention provides isolated nucleic acid molecules encoding CARD-12 proteins or biologically active portions thereof, as well as nucleic acid fragments suitable as primers or hybridization probes for the detection of CARD-12 encoding nucleic acids.

The invention encompasses methods of diagnosing and treating patients who are suffering from a disorder associated with an abnormal level or rate (undesirably high or undesirably low) of apoptotic cell death, abnormal activity of stress-related pathways of the endoplasmic reticulum (ER), abnormal activity of the Fas/APO-1 receptor complex, abnormal activity of the TNF receptor complex, or abnormal activity of a caspase by administering a compound that modulates the expression of CARD-12 (at the DNA, mRNA or protein level, e.g., by altering mRNA splicing) or by altering the activity of CARD-12. Examples of such compounds include small molecules, antisense nucleic acid molecules, ribozymes, and polypeptides.

Certain disorders are associated with an increased number of surviving cells, which are produced and continue to survive or proliferate when apoptosis is inhibited or occurs at an undesirably low rate. CARD-12 and compounds that modulate the expression or activity of CARD-12 can be used to treat or diagnose such disorders. These disorders include cancer (particularly follicular lymphomas, chronic myelogenous leukemia, melanoma, colon cancer, lung carcinoma, carcinomas associated with mutations in p53, and hormone-dependent tumors such as breast cancer, prostate cancer, and ovarian cancer). Such compounds can also be used to treat viral infections (such as those caused by herpesviruses, poxviruses, and adenoviruses). Failure to remove autoimmune cells that arise during development or that develop as a result of somatic mutation during an immune response can result in autoimmune disease. Thus, an autoimmune disorder can be caused by an undesirably low level of apoptosis. Accordingly, CARD-12 and modulators of CARD-12 activity or expression can be used to treat autoimmune disorders (e.g., systemic lupus erythematosus, immune-mediated glomerulonephritis, and arthritis).

Many diseases are associated with an undesirably high rate of apoptosis. CARD-12 and modulators of CARD-12 expression or activity can be used to treat or diagnose such disorders. A wide variety of neurological diseases are characterized by the gradual loss of specific sets of neurons. Such disorders include Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), retinitis pigmentosa, spinal muscular atrophy, Huntington's disease, and various forms of cerebellar degeneration. The cell loss in these diseases does not induce an inflammatory response, and apoptosis appears to be the mechanism of cell death. In addition, a number of hematologic diseases are associated with a decreased production of blood cells. These disorders include anemia associated with chronic disease, aplastic anemia, chronic neutropenia, and the myelodysplastic syndromes. Disorders of blood cell production, such as myelodysplastic syndrome and some forms of aplastic anemia, are associated with increased apoptotic cell

death within the bone marrow. These disorders could result from the activation of genes that promote apoptosis, acquired deficiencies in stromal cells or hematopoietic survival factors, or the direct effects of toxins and mediators of immune responses. Two common disorders associated with cell death are myocardial infarctions and stroke. In both disorders, cells within the central area of ischemia, which is produced in the event of acute loss of blood flow, appear to die rapidly as a result of necrosis. However, outside the central ischemic zone, cells die over a more protracted time period and morphologically appear to die by apoptosis. Additional diseases associated with an undesirably high rate of apoptosis include: ischemic and hypoxic brain injury, traumatic and excitotoxic brain damage, neuronal transplantation, acute bacterial meningitis, kidney ischemia/reperfusion injury, and liver disease. CARD-12 and modulators of CARD-12 may therefore be useful in treating and diagnosing these conditions.

Populations of cells are often depleted in the event of viral infection, with perhaps the most dramatic example being the cell depletion caused by the human immunodeficiency virus (HIV). Surprisingly, most T cells that die during HIV infections do not appear to be infected with HIV. Although a number of explanations have been proposed, recent evidence suggests that stimulation of the CD4 receptor results in the enhanced susceptibility of uninfected T cells to undergo apoptosis.

CARD-12 polypeptides, nucleic acids and modulators of CARD-12 expression or activity can be used to treat inflammatory disorders and immune system disorders. The inflammatory and immune disorders include, but are not limited to, chronic inflammatory diseases and disorders, such as Crohn's disease, reactive arthritis, including Lyme disease, insulin-dependent diabetes, organ-specific autoimmunity, including multiple sclerosis, Hashimoto's thyroiditis and Grave's disease, contact dermatitis, psoriasis, graft rejection, graft versus host disease, sarcoidosis, atopic conditions, such as asthma and allergy, including allergic rhinitis, gastrointestinal allergies, including food allergies, eosinophilia, conjunctivitis, glomerular nephritis, certain pathogen susceptibilities such as helminthic (e.g., leishmaniasis), certain viral infections, including HIV, and bacterial infections, including tuberculosis and lepromatous leprosy.

Ischemia is often accompanied by inflammation that causes cell death. Because CARD-12 is expected to play a role in stress-related response, inflammation and apoptosis, CARD-12 polypeptides, nucleic acids, and modulators of CARD-12 expression or activity can be used to treat cells death accompanying inflammatory responses triggered by ischemia.

Invasive infection with Gram-negative bacteria and Gram-positive bacteria often results in septic shock. CARD-12 may recognize and bind components of Gram-

negative bacteria and Gram-positive bacteria or other infectious agents (e.g., intracellular parasites), triggering an inflammatory response. Thus, CARD-12 may play a role in innate immune system responses that is similar to that of Toll-like receptor 2 (TLR2), a receptor which has some structural similarity to plant R proteins and IL-1R. TLR2 is a signaling receptor that, in association with CD14, is activated by LPS in a response that requires LPS-binding protein. The interaction of TLR2 with LPS leads to TLR2 oligomerization and recruitment of IRAK (Yang et al. (1998) *Nature* 395:284-88; Yang et al (1999) *J. Immunol.* 163:639-43; and Yoshimura et al. (1999) *J. Immunol.* 163:105). Thus, TLR2 is thought to be a direct mediator of signaling by LPS. TLR2 is also thought to mediate cell activation induced by peptidoglycan and lipoteichoic acid, the main stimulatory components of Gram-positive bacteria (Schwandner et al. (1999) *J. Biol. Chem.* 274:17406-09).

In addition to the aforementioned disorders, CARD-12 polypeptides, nucleic acids, and modulators of CARD-12 expression or activity can be used to treat septic shock and other disorders associated with an innate immune response. For example, CARD-12 may bind to a component of an intracellular infectious agent or a component of an infectious agent that is brought into a cell expressing CARD-12, e.g., a component that enters a cell through a receptor or is expressed by a viral gene.

In addition to the aforementioned disorders, CARD-12 polypeptides, nucleic acids, and modulators of CARD-12 expression or activity can be used to treat disorders of cell signaling and disorders of tissues in which CARD-12 is expressed.

The invention features a nucleic acid molecule which is at least 45% (or 55%, 65%, 75%, 85%, 95%, or 98%) identical to the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, the nucleotide sequence of the cDNA insert of the plasmid deposited with the ATCC as Accession Number _____ (the "cDNA of ATCC _____"), or a complement thereof.

The invention features a nucleic acid molecule which includes a fragment of at least 150 (300, 325, 350, 375, 400, 425, 450, 500, 550, 600, 650, 700, 800, 900, 1000, 1300, 1600, 1900, 2100, 2400, 2700, 3000, or 3100) nucleotides of the nucleotide sequence shown in SEQ ID NO:1, or SEQ ID NO:3, or the nucleotide sequence of the cDNA ATCC _____, or a complement thereof.

In an embodiment, a CARD-12 nucleic acid molecule has the nucleotide sequence shown in SEQ ID NO:1, or SEQ ID NO:3, or the nucleotide sequence of the cDNA of ATCC _____.

Also within the invention is a nucleic acid molecule which encodes a fragment of a polypeptide having the amino acid sequence of SEQ ID NO:2 or the polypeptide encoded by the cDNA of ATCC Accession Number _____.

5 The invention includes a nucleic acid molecule which encodes a naturally occurring allelic variant of a polypeptide comprising the amino acid sequence of SEQ ID NO:2, wherein the nucleic acid molecule hybridizes to a nucleic acid molecule consisting of SEQ ID NO:1, SEQ ID NO:3, or the cDNA of ATCC under stringent conditions.

In general, an allelic variant of a gene will be readily identifiable as mapping to the same chromosomal location as the gene.

10 The invention also includes a nucleic acid molecule encoding a naturally occurring polypeptide, wherein the nucleic acid hybridizes to a nucleic acid molecule consisting of SEQ ID NO:3 under stringent conditions (e.g., hybridization in 6X sodium chloride/sodium citrate (SSC) at about 60°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 65°C), and wherein the nucleic acid encodes a polypeptide of 1020-
15 1028 amino acids in length, preferably 1024 amino acids, having a molecular weight of about 116.1 kD prior to post-translational modifications. Thus, the invention encompasses a nucleic acid molecule which includes the sequence of the protein coding region of a naturally occurring mRNA (or the corresponding cDNA sequence) that is expressed in a human cell.

20 Also within the invention are: an isolated CARD-12 protein having an amino acid sequence that is at least about 65%, preferably 75%, 85%, 95%, or 98% identical to the amino acid sequence of SEQ ID NO:2; an isolated CARD-12 protein having an amino acid sequence that is at least about 85%, 95%, or 98% identical to the P-loop domain of SEQ ID NO:2 (e.g., about amino acid residues 169 to 179 of SEQ ID NO:2); an isolated
25 CARD-12 protein having an amino acid sequence that is at least about 85%, 95%, or 98% identical to the CARD domain of SEQ ID NO:2 (e.g., about amino acid residues 1 to 88 of SEQ ID NO:2); an isolated CARD-12 protein having an amino acid sequence that is at least about 65%, preferably 75%, 85%, 95%, or 98% identical to the nucleotide binding site core domain of SEQ ID NO:2 (e.g., about amino acid residues 161 to 323 of SEQ ID
30 NO:2); an isolated CARD-12 protein having an amino acid sequence that is at least about 65%, preferably 75%, 85%, 95%, or 98% identical to one or more of the leucine rich repeats of SEQ ID NO:2 (e.g., about amino acids residues 762-789, 819-846, 847-874, and 938-965 of SEQ ID NO:2); and an isolated CARD-12 protein having an amino acid sequence that is at least about 85%, 95%, or 98% identical to the NAIP homology region
35 of SEQ ID NO:2 (e.g., about amino acid residues 150 to 1024 of SEQ ID NO:2).

Also within the invention are: an isolated CARD-12 protein which is encoded by a nucleic acid molecule having a nucleotide sequence that is at least about 65%, preferably 75%, 85%, or 95% identical to SEQ ID NO:3 or the cDNA of ATCC _____; an isolated CARD-12 protein which is encoded by a nucleic acid molecule having a nucleotide sequence at least about 85%, 95%, or 98% identical to the P-loop domain encoding portion of SEQ ID NO:3; an isolated CARD-12 protein which is encoded by a nucleic acid molecule having a nucleotide sequence at least about 65% preferably 75%, 85%, or 95% identical the CARD domain encoding portion of SEQ ID NO:3; an isolated CARD-12 protein which is encoded by a nucleic acid molecule having a nucleotide sequence at least about 65% preferably 75%, 85%, or 95% identical the NAIP homology encoding portion of SEQ ID NO:3; an isolated CARD-12 protein which is encoded by a nucleic acid molecule having a nucleotide sequence at least about 65% preferably 75%, 85%, or 95% identical the nucleotide binding site domain encoding portion of SEQ ID NO:3; an isolated CARD-12 protein which is encoded by a nucleic acid molecule having a nucleotide sequence at least about 65% preferably 75%, 85%, or 95% identical to the LRR domain encoding portion of SEQ ID NO:3 or one or more leucine rich repeat encoding portions of SEQ ID NO:3; and an isolated CARD-12 protein which is encoded by a nucleic acid molecule having a nucleotide sequence which hybridizes under stringent hybridization conditions to a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:3 or the non-coding strand of the cDNA of ATCC _____.

The CARD-12 nucleic acids, polypeptides, and antibodies of the invention may be useful for mapping the location of the CARD-12 gene.

Another embodiment of the invention features CARD-12 nucleic acid molecules which specifically detect CARD-12 nucleic acid molecules, relative to nucleic acid molecules encoding other members of the CARD superfamily and/or members of the NBS/LRR superfamily. For example, in one embodiment, a CARD-12 nucleic acid molecule hybridizes under stringent conditions to a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, or the cDNA of ATCC _____, or a complement thereof. In another embodiment, the CARD-12 nucleic acid molecule is at least 300 (350, 400, 450, 500, 550, 600, 650, 700, 800, 900, 1000, 1300, 1600, 1900, 2100, 2400, 2700, 3000, or 3100) nucleotides in length and hybridizes under stringent conditions to a nucleic acid molecule comprising the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, the cDNA of ATCC _____, or a complement thereof. In another embodiment, an isolated CARD-12 nucleic acid molecule comprises the CARD domain encoding portion of SEQ ID NO:3, or a complement thereof. In yet another embodiment,

the invention provides an isolated nucleic acid molecule which is antisense to the coding strand of a CARD-12 nucleic acid.

Another aspect of the invention provides a vector, e.g., a recombinant expression vector, comprising a CARD-12 nucleic acid molecule of the invention. In another embodiment the invention provides a host cell containing such a vector. The invention also provides a method for producing CARD-12 protein by culturing, in a suitable medium, a host cell of the invention containing a recombinant expression vector such that a CARD-12 protein is produced.

Another aspect of this invention features isolated or recombinant CARD-12 proteins and polypeptides. Preferred CARD-12 proteins and polypeptides possess at least one biological activity possessed by naturally occurring human CARD-12, e.g., (1) the ability to form protein:protein interactions with proteins in the apoptotic signaling pathway; (2) the ability to form CARD-CARD interactions with proteins in the apoptotic signaling pathway; (3) the ability to bind a CARD-12 ligand; and (4) the ability to bind to an intracellular target. Other activities include: (1) modulation of cellular proliferation; (2) modulation of cellular differentiation; (3) modulation of cellular death; (4) modulation of ER-specific apoptosis pathways; (5) modulation of the NF- κ B pathway; (6) modulation of stress-responsive signaling pathways; and (7) modulation of an innate immune response.

The CARD-12 proteins of the present invention, or biologically active portions thereof, can be operatively linked to a non-CARD-12 polypeptide (e.g., heterologous amino acid sequences) to form CARD-12 fusion proteins, respectively. The invention further features antibodies that specifically bind CARD-12 proteins, such as monoclonal or polyclonal antibodies. In addition, the CARD-12 proteins or biologically active portions thereof can be incorporated into pharmaceutical compositions, which optionally include pharmaceutically acceptable carriers.

In another aspect, the present invention provides a method for detecting the presence of CARD-12 activity or expression in a biological sample by contacting the biological sample with an agent capable of detecting an indicator of CARD-12 activity such that the presence of CARD-12 activity is detected in the biological sample.

In another aspect, the invention provides a method for modulating CARD-12 activity comprising contacting a cell with an agent that modulates (inhibits or stimulates) CARD-12 activity or expression such that CARD-12 activity or expression in the cell is modulated. In one embodiment, the agent is an antibody that specifically binds to CARD-12 protein. In another embodiment, the agent modulates expression of CARD-12 by modulating transcription of a CARD-12 gene, splicing of a CARD-12 mRNA, or

translation of a CARD-12 mRNA. In yet another embodiment, the agent is a nucleic acid molecule having a nucleotide sequence that is antisense to the coding strand of the CARD-12 mRNA or the CARD-12 gene.

In one embodiment, the methods of the present invention are used to treat a subject having a disorder characterized by aberrant CARD-12 protein or nucleic acid expression or activity or related to CARD-12 expression or activity by administering an agent which is a CARD-12 modulator to the subject. In one embodiment, the CARD-12 modulator is a CARD-12 protein. In another embodiment the CARD-12 modulator is a CARD-12 nucleic acid molecule. In other embodiments, the CARD-12 modulator is a peptide, peptidomimetic, or other small molecule.

The present invention also provides a diagnostic assay for identifying the presence or absence of a genetic lesion or mutation characterized by at least one of: (i) aberrant modification or mutation of a gene encoding a CARD-12 protein; (ii) mis-regulation of a gene encoding a CARD-12 protein; (iii) aberrant RNA splicing; and (iv) aberrant post-translational modification of a CARD-12 protein, wherein a wild-type form of the gene encodes a protein with a CARD-12 activity.

In another aspect, the invention provides a method for identifying a compound that binds to or modulates the activity of a CARD-12 protein. In general, such methods entail measuring a biological activity of a CARD-12 protein in the presence and absence of a test compound and identifying those compounds which alter the activity of the CARD-12 protein.

The invention also features methods for identifying a compound which modulates the expression of CARD-12 by measuring the expression of CARD-12 in the presence and absence of a compound.

The invention also features methods for treating disorders associated with inappropriate apoptosis (e.g., Alzheimer's diseases or other neurological disorders associated with neuronal apoptosis) by modulating the expression or activity of CARD-12.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

Brief Description of the Drawings

Figures 1A-1E depict the cDNA sequence (SEQ ID NO:1) and predicted amino acid sequence (SEQ ID NO:2) of human CARD-12. The open reading frame of CARD-12 (SEQ ID NO:1) extends from nucleotide 36 to nucleotide 3107 of SEQ ID NO:1 (SEQ ID NO:3).

Sub
Q1

Figures 2A-2E depict a predicted nucleotide sequence (SEQ ID NO:4) and predicted amino acid sequence (SEQ ID NO:5) of human CARD-12, based upon the ~~CARD-12 sequence derived from a genomic clone.~~

Figure 3 depicts a hydropathy plot of CARD-12. Relatively hydrophobic residues are above the dashed horizontal line, and relatively hydrophilic residues are below the dashed horizontal line. The cysteine residues (cys) and N-glycosylation (Ngly) site are indicated by short vertical lines just below the hydropathy trace.

Figure 4 depicts a plot showing the predicted structural features of CARD-12. This figure shows the predicted alpha regions (Garnier-Robson and Chou-Fasman), the predicted beta regions (Garnier-Robson and Chou-Fasman), the predicted turn regions (Garnier-Robson and Chou-Fasman) and the predicted coil regions (Garnier-Robson). Also included in the figure is a hydrophilicity plot (Kyte-Doolittle), the predicted alpha and beta-amphipathic regions (Eisenberg), the predicted flexible regions (Karplus-Schulz), the predicted antigenic index (Jameson-Wolf) and the predicted surface probability plot (Emini).

Figure 5A depicts an alignment of amino acids 2-88 of human CARD-12 (amino acid residues 2-88 of SEQ ID NO:2) with a CARD domain (SEQ ID NO:7) derived from a hidden Markov model.

Figure 5B depicts an alignment of amino acids 764-791 of human CARD-12 (amino acid residues 764-791 of SEQ ID NO:2) with a consensus leucine rich repeat (SEQ ID NO:8) derived from a hidden Markov model.

Figure 5C depicts an alignment of amino acids 821-848 of human CARD-12 (amino acid residues 821-848 of SEQ ID NO:2) with a consensus leucine rich repeat (SEQ ID NO:8) derived from a hidden Markov model.

Figure 5D depicts an alignment of amino acids 849-872 of human CARD-12 (amino acid residues 849-872 of SEQ ID NO:2) with a consensus leucine rich repeat (SEQ ID NO:8) derived from a hidden Markov model.

Figure 5E depicts an alignment of amino acids 938-965 of human CARD-12 (amino acid residues 938-965 of SEQ ID NO:2) with a consensus leucine rich repeat (SEQ ID NO:8) derived from a hidden Markov model.

Sub
Q2

Figures 6A-6C depict an alignment of amino acids 150-1024 of human CARD-12 (amino acid residues 150-1024 of SEQ ID NO:2) with amino acids 451-1232 of neuronal AIP (SEQ ID NO:9).

Detailed Description of the Invention

The present invention is based, in part, on the identification of a cDNA sequence encoding a human CARD-12 protein. A nucleotide sequence encoding a human CARD-12 protein is shown in Figures 1A-1E (SEQ ID NO:1; SEQ ID NO:3 includes the open reading frame only). A predicted amino acid sequence of CARD-12 protein is also shown in Figures 1A-1E (SEQ ID NO:2).

A BAC clone (GenBank™ Accession Number AL121653) was searched to identify potential exons. The predicted protein sequences were then searched using a profile of known CARD domains. This search led to the identification of a sequence predicted to encode a CARD domain-containing protein later identified as CARD-12. Figures 2A-2E depict a nucleotide sequence (SEQ ID NO:4) assembled from the BAC clone which includes a predicted open reading frame (SEQ ID NO:6; nucleotides 1-3612 of SEQ ID NO:4) encoding a 1204 amino acid protein (SEQ ID NO:5).

The CARD-12 sequence assembled from the BAC clone was used to further characterize the CARD-12 cDNA sequence. A search of a proprietary electronic cDNA database (created from a human lymphocyte library) using sequences derived from the 3' end of SEQ ID NO:4 led to the identification of a cDNA containing the 3' end of the CARD-12 cDNA. The sequence of the 5' end of the CARD-12 cDNA was determined by a search of the Incyte (Palo Alto, CA) Life Gold Templates cDNA electronic database using sequences from the 5' end of SEQ ID NO:4. A cDNA sequence was identified (Incyte clone number 328193) that contained 366 nucleotides of the 5' portion of the CARD-12 cDNA. Primers were designed corresponding to the 5' untranslated region and the 3' carboxy terminus coding region of CARD-12. PCR amplification using these primers was performed on a placenta cDNA library. The sequencing of PCR products led to the identification of the cDNA sequence of CARD-12 (SEQ ID NO:1).

The CARD-12 cDNA sequence (SEQ ID NO:1) has some differences compared to that predicted by the assembly of the predicted CARD-12 exons of the BAC clone (SEQ ID NO:4). These differences are generally located at the 5' and 3' end of the cDNA. Where the CARD-12 sequences depicted in SEQ ID NO:1 and SEQ ID NO:4 differ, SEQ ID NO:1 corresponds to the CARD-12 cDNA sequence.

Figures 1A-1E depict the sequence of a 3133 nucleotide cDNA (SEQ ID NO:1) which includes a predicted open reading frame (SEQ ID NO:3; nucleotides 36-3107 of SEQ ID NO:1) encoding a 1024 amino acid human CARD-12 protein (SEQ ID NO:2). Human CARD-12 is predicted to be an intracellular protein.

The predicted amino acid sequence of human CARD-12 was compared to amino acid sequences of known proteins and various motifs were identified. The 1024 amino

acid human CARD-12 protein includes two N-glycosylation sites (e.g., about amino acid residues 539-542 and 764-767 of SEQ ID NO:2); a glycosaminoglycan attachment site (e.g., about amino acid residues 171-174 of SEQ ID NO:2); four cAMP- and cGMP-dependent protein kinase phosphorylation site (e.g., about amino acid residues 60-63, 228-231, 453-456, and 985-988 of SEQ ID NO:2); 11 protein kinase C phosphorylation sites (e.g., about amino acid residues 72-74, 171-173, 188-190, 226-228, 403-405, 536-538, 566-568, 665-667, 689-691, 710-712, and 973-975 of SEQ ID NO:2); 20 casein kinase II phosphorylation sites (e.g., about amino acid residues 72-75, 94-97, 133-136, 215-218, 279-282, 365-368, 415-418, 445-448, 460-463, 479-482, 497-500, 541-544, 553-556, 607-610, 665-668, 725-728, 742-745, 851-854, 920-923, and 973-976 of SEQ ID NO:2); a tyrosine kinase phosphorylation site (e.g., about amino acid residues 71-78 of SEQ ID NO:2); 15 N-myristoylation sites (e.g., about amino acid residues 62-67, 156-161, 187-192, 211-216, 291-296, 380-385, 516-521, 618-623, 699-704, 754-759, 760-765, 894-899, 928-933, 946-951, and 959-964 of SEQ ID NO:2); an amidation site (e.g., about amino acid residues 451-454 of SEQ ID NO:2); and an ATP/GTP-binding site motif A (P-loop) (e.g., about amino acid residues 169-179 of SEQ ID NO:2).

Figure 3 depicts a hydropathy plot of CARD-12. Relatively hydrophobic residues are above the dashed horizontal line, and relatively hydrophilic residues are below the dashed horizontal line. The cysteine residues (cys) and N-glycosylation (Ngly) site are indicated by short vertical lines just below the hydropathy trace.

A plot showing the predicted structural features of CARD-12 is presented in Figure 4. This figure shows the predicted alpha regions (Garnier-Robson and Chou-Fasman), the predicted beta regions (Garnier-Robson and Chou-Fasman), the predicted turn regions (Garnier-Robson and Chou-Fasman) and the predicted coil regions (Garnier-Robson). Also included in the figure is a hydrophilicity plot (Kyte-Doolittle), the predicted alpha and beta-amphipathic regions (Eisenberg), the predicted flexible regions (Karplus-Schulz), the predicted antigenic index (Jameson-Wolf) and the predicted surface probability plot (Emini).

An analysis of the predicted CARD-12 amino acid sequence showed it to contain a CARD domain (e.g., about amino acid residues 1-88 of SEQ ID NO:2), a nucleotide binding site (NBS) domain (e.g., about amino acid residues 161-323 of SEQ ID NO:2), and three leucine rich repeats (LRR; e.g., about amino acid residues 762-789, 819-846, 847-874, and 938-965 of SEQ ID NO:2) which form a LRR domain (e.g., about amino acid residues 762-965 of SEQ ID NO:2). Within the predicted NBS domain there is a kinase 1a domain (P-loop) (e.g., about amino acid residues 169-179 of SEQ ID NO:2) and a kinase 2 domains (e.g., about amino acid residues 245-248 of SEQ ID NO:2).

Figure 5A depicts an alignment of amino acids 2-88 of human CARD-12 (amino acid residues 2-88 of SEQ ID NO:2) with a consensus CARD domain (SEQ ID NO:7) derived from a HMM.

Figures 5B-5E each depict an alignment of one of the four leucine rich repeats within the LRR domain of CARD-12 (amino acid residues 764-791 of SEQ ID NO:2 (Figure 5B), amino acid residues 821-848 of SEQ ID NO:2 (Figure 5C), amino acid residues 849-872 of SEQ ID NO:2 (Figure 5D), and amino acid residues 938-965 of SEQ ID NO:2 (Figure 5E)) with a consensus LRR (SEQ ID NO:8) derived from a HMM.

The domain alignments depicted in Figures 5A-5E were identified by homology searching using consensus domains derived from hidden Markov models (HMMs). HMMs can be used to perform multiple sequence alignment and very sensitive database searching, using statistical descriptions of a domain's consensus sequence. For more information on HMM searches, see, e.g., <http://hmmer.wustl.edu/>. In the alignments of Figures 5A-5E a single letter amino acid designation at a position on the line between the CARD-12 sequence and the HMM-generated consensus domain sequence indicates an exact match between the two. A "+" in this middle line indicates a conservative substitution at the particular residue of CARD-12. Amino acid residues located in the domains identified by the HMM search may be important for the appropriate functioning of the CARD-12 protein. For this reason, amino acid substitutions with respect to the sequence of SEQ ID NO:2 that are outside of the domains homologous to HMM consensus domains may be less detrimental to the activity of the CARD-12 protein.

The C-terminal portion of CARD-12 (amino acids 150 to 1024 of SEQ ID NO:2) bears some similarity to the N-terminus of neuronal AIP (GenBank™ Accession Number Q13075; Roy et al. (1995) Cell 80:167-178). Figures 6A-6C depict an alignment of amino acids 451-1232 of neuronal AIP (SEQ ID NO:9) and amino acids 150-1024 of human CARD-12.

TABLE 1: Summary of Human CARD-12 Sequence Information

Source of CARD-12 Sequence	Predicted cDNA	Predicted Protein	Predicted ORF	Figure
cDNA sequence	SEQ ID NO:1	SEQ ID NO:2	SEQ ID NO:3	Figs. 1A-1E
genomic sequence	SEQ ID NO:4	SEQ ID NO:5	SEQ ID NO:6	Figs. 2A-2E

TABLE 2: Summary of Domains of CARD-12

Domain	Location in CARD-12
CARD	about amino acids 1-88 of SEQ ID NO:2
NBS	about amino acids 161-323 of SEQ ID NO:2
Kinase 1a domain (P-loop)	about amino acids 169-179 of SEQ ID NO:2
Kinase 2 domain (Walker B box)	about amino acids 245-248 of SEQ ID NO:2
Neuronal AIP Homology	about amino acids 150-1024 of SEQ ID NO:2
Leucine rich repeats	about amino acids 762-789, 819-846, 847-874, and 938-965 of SEQ ID NO:2
LRR	about amino acids 762-965 of SEQ ID NO:2

A plasmid containing a ___ encoding human CARD-12 (p___) was deposited with the American Type Culture Collection (ATCC), Manassas, VA on ___, 1999, and assigned Accession Number _____. This deposit will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. This deposit was made merely as a convenience for those of skill in the art and is not an admission that a deposit is required under 35 U.S.C. §112.

A region, the CARD domain, of human CARD-12 protein (SEQ ID NO:2) bears some similarity to the CARD domains of CARD-3, CARD-4, CARD-5, CARD-6, CARD-7, CARD-8, CARD-9, CARD-10, CARD-11, CARD-13, CARD-14, and CARD-15. Detailed information concerning CARD-3, CARD-4, CARD-5, CARD-6, CARD-7, CARD-8, CARD-9, CARD-10, CARD-11, CARD-13, CARD-14, and CARD-15, can be found in U.S. Application Serial No. 09/245,281, filed February 5, 1999, U.S. Application Serial No. 09/207,359, filed December 8, 1998, U.S. Application Serial No. 09/099,041, filed June 17, 1998, U.S. Application Serial No. 09/019,942, filed February 6, 1998, U.S. Application Serial No. 09/428,252, filed October 27, 1999, U.S. Application Serial No. 60/180,021, filed February 3, 2000, U.S. Application Serial No. 09/573,641, filed May 17, 2000, U.S. Application Serial No. 60/181,159 filed February 9, 2000, U.S. Application Serial No. 60/168,780 filed December 3, 1999, U.S. Application Serial No. 09/507,533 filed February 18, 2000, and U.S. Application Serial No. 09/513,904 filed February 25, 2000. The entire content of each of these applications is incorporated herein by reference.

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ability to interact directly or indirectly with a Bcl-2 family member; (ix) the ability to modulate the activity of a stress activated kinase (e.g., JNK/p38); and (x) the ability to modulate phosphorylation of CHOP (GADD 153). CARD-12 nucleic acid and polypeptides as well as modulators of activity of expression of CARD-12 might be used to modulate an Apaf-1 signaling pathway. CARD-12 may modulate the activity of a neurotrophin receptor and thus modulate apoptosis of neuronal cells. Accordingly, CARD-12 nucleic acids and polypeptides as well as modulators of CARD-12 activity or expression can be used to modulate apoptosis of neurons (e.g., for treatment of neurological disorders, particularly neurodegenerative disorders).

Accordingly, another embodiment of the invention features isolated CARD-12 proteins and polypeptides having a CARD-12 activity.

Various aspects of the invention are described in further detail in the following subsections.

I. Isolated Nucleic Acid Molecules

One aspect of the invention pertains to isolated nucleic acid molecules that encode CARD-12 proteins or biologically active portions thereof, as well as nucleic acid molecules sufficient for use as hybridization probes to identify CARD-12-encoding nucleic acids (e.g., CARD-12 mRNA) and fragments for use as PCR primers for the amplification or mutation of CARD-12 nucleic acid molecules. As used herein, the term "nucleic acid molecule" is intended to include DNA molecules (e.g., cDNA or genomic DNA) and RNA molecules (e.g., mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

An "isolated" nucleic acid molecule is one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. Preferably, an "isolated" nucleic acid is free of sequences (preferably protein encoding sequences) that which naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated CARD-12 nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

A nucleic acid molecule of the present invention, e.g., a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, the cDNA of ATCC _____, or a complement of any of these nucleotide sequences, can be isolated using standard molecular biology techniques and the sequence information provided herein.

5 Using all or portion of the nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:3, the cDNA of ATCC _____, as a hybridization probe, CARD-12 nucleic acid molecules can be isolated using standard hybridization and cloning techniques (e.g., as described in Sambrook et al., eds., Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 10 1989).

A nucleic acid of the invention can be amplified using cDNA, mRNA or genomic DNA as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides 15 corresponding to CARD-12 nucleotide sequences can be prepared by standard synthetic techniques, e.g., using an automated DNA synthesizer.

In another embodiment, an isolated nucleic acid molecule of the invention comprises a nucleic acid molecule which is a complement of the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, the cDNA of ATCC _____, or a portion 20 thereof. A nucleic acid molecule which is complementary to a given nucleotide sequence is one which is sufficiently complementary to the given nucleotide sequence that it can hybridize to the given nucleotide sequence thereby forming a stable duplex.

Moreover, the nucleic acid molecule of the invention can comprise only a portion 25 of a nucleic acid sequence encoding CARD-12, for example, a fragment which can be used as a probe or primer or a fragment encoding a biologically active portion of CARD-12. The nucleotide sequence determined from the cloning of the human CARD-12 gene allows for the generation of probes and primers designed for use in identifying and/or cloning CARD-12 homologues in other cell types, e.g., from other tissues, as well as CARD-12 homologues and orthologs from other mammals. The probe/primer typically 30 comprises substantially purified oligonucleotide. The oligonucleotide typically comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least about 12, preferably about 25, more preferably about 50, 75, 100, 125, 150, 175, 200, 250, 300, 350 or 400 consecutive nucleotides of the sense or anti-sense sequence of SEQ ID NO:1, SEQ ID NO:3, the cDNA of ATCC _____, or of a naturally occurring 35 mutant of one of SEQ ID NO:1, SEQ ID NO:3, or the cDNA of ATCC _____.

Probes based on the CARD-12 nucleotide sequence can be used to detect transcripts or genomic sequences encoding the same or similar proteins. The probe comprises a label group attached thereto, e.g., a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor. Such probes can be used as a part of a diagnostic test kit for identifying allelic variants and orthologs of the CARD-12 proteins of the present invention, identifying cells or tissue which mis-express a CARD-12 protein, such as by measuring a level of a CARD-12-encoding nucleic acid in a sample of cells from a subject, e.g., detecting CARD-12 mRNA levels or determining whether a genomic CARD-12 gene has been mutated or deleted.

A nucleic acid fragment encoding a "biologically active portion" of CARD-12 can be prepared by isolating a portion of SEQ ID NO:1, SEQ ID NO:3, or the cDNA of ATCC _____, which encodes a polypeptide having a CARD-12 biological activity, expressing the encoded portion of CARD-12 protein (e.g., by recombinant expression in vitro) and assessing the activity of the encoded portion of CARD-12. For example, a nucleic acid fragment encoding a biologically active portion of CARD-12 includes a CARD domain, e.g., amino acids 1-88 of SEQ ID NO:2.

The invention further encompasses nucleic acid molecules that differ from the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, and the cDNA of ATCC _____, due to degeneracy of the genetic code and thus encode the same CARD-12 protein as that encoded by the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, or the cDNA of ATCC _____.

In addition to the CARD-12 nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, and the cDNA of ATCC _____, it will be appreciated by those skilled in the art that DNA sequence polymorphisms that lead to changes in the amino acid sequences of CARD-12 may exist within a population (e.g., the human population). Such genetic polymorphism in the CARD-12 gene may exist among individuals within a population due to natural allelic variation. As used herein, the terms "gene" and "recombinant gene" refer to nucleic acid molecules comprising an open reading frame encoding a CARD-12 protein, preferably a mammalian CARD-12 protein. Such natural allelic variations can typically result in 1-5% variance in the nucleotide sequence of the CARD-12 gene. Any and all such nucleotide variations and resulting amino acid polymorphisms in CARD-12 that are the result of natural allelic variation and that do not alter the functional activity of CARD-12 are intended to be within the scope of the invention. Thus, e.g., 1%, 2%, 3%, 4%, or 5% of the amino acids in CARD-12 (e.g., 1, 2, 3, 4, 5, 6, 8, 10, 15, or 20 amino acids) are replaced by another amino acid, preferably by conservative substitution.

Moreover, nucleic acid molecules encoding CARD-12 proteins from other species (CARD-12 orthologs/homologues), which have a nucleotide sequence which differs from that of a CARD-12 disclosed herein, are intended to be within the scope of the invention.

Accordingly, in another embodiment, an isolated nucleic acid molecule of the invention is at least 150 (300, 325, 350, 375, 400, 425, 450, 500, 550, 600, 650, 700, 800, 900, 1000, 1300, 1600, 1900, 2100, 2400, 2700, 3000, or 3100) nucleotides in length and hybridizes under stringent conditions to the nucleic acid molecule comprising the nucleotide sequence, preferably the coding sequence, of SEQ ID NO:1, SEQ ID NO:3, or the cDNA of ATCC _____.

As used herein, the term "hybridizes under stringent conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences at least 60% (65%, 70%, preferably 75%) identical to each other typically remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. An, non-limiting example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 50-65 C (e.g., 50°C or 60°C or 65°C). Preferably, the isolated nucleic acid molecule of the invention that hybridizes under stringent conditions corresponds to a naturally-occurring nucleic acid molecule. As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in a human cell in nature (e.g., encodes a natural protein).

In addition to naturally-occurring allelic variants of the CARD-12 sequence that may exist in the population, the skilled artisan will further appreciate that changes can be introduced by mutation into the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, or the cDNA of ATCC _____, thereby leading to changes in the amino acid sequence of the encoded protein without altering the functional ability of the protein. For example, one can make nucleotide substitutions leading to amino acid substitutions at "non-essential" amino acid residues. A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of CARD-12 protein without altering the biological activity, whereas an "essential" amino acid residue is required for biological activity. For example, amino acid residues that are conserved among the CARD-12, proteins of various species are predicted to be particularly unamenable to alteration.

For example, preferred CARD-12 proteins of the present invention contain at least one CARD domain. Additionally, a CARD-12 protein also contains at least one kinase-2 domain, at least one P-loop domain, at least one nucleotide binding site domain, and at

least one LRR domain. Such conserved domains are less likely to be amenable to mutation. Other amino acid residues, however, (e.g., those that are not conserved or only semi-conserved among CARD-12 of various species) may not be essential for activity and thus are likely to be amenable to alteration.

Accordingly, another aspect of the invention pertains to nucleic acid molecules encoding CARD-12 proteins that contain changes in amino acid residues that are not essential for activity. Such CARD-12 proteins differ in amino acid sequence from SEQ ID NO:2, and yet retain biological activity. In one embodiment, the isolated nucleic acid molecule includes a nucleotide sequence encoding a protein that includes an amino acid sequence that is at least about 45% identical, 65%, 75%, 85%, 95%, or 98% identical to the amino acid sequence of SEQ ID NO:2. An isolated nucleic acid molecule encoding a CARD-12 protein having a sequence which differs from that of SEQ ID NO:1, SEQ ID NO:3, or cDNA of ATCC _____, can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of CARD-12 (SEQ ID NO:1, SEQ ID NO:3, the cDNA of ATCC _____) such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations can be introduced by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues. Thus, for example, 1%, 2%, 3%, 5%, or 10% of the amino acids can be replaced by conservative substitution. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in CARD-12 is preferably replaced with another amino acid residue from the same side chain family. Alternatively, mutations can be introduced randomly along all or part of a CARD-12 coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for CARD-12 biological activity to identify mutants that retain activity. Following mutagenesis, the encoded protein can be expressed recombinantly and the activity of the protein can be determined.

In an embodiment, a mutant CARD-12 protein can be assayed for: (1) the ability to form protein:protein interactions with proteins in the apoptotic signaling pathway; (2) the ability to bind a CARD-12 ligand; or (3) the ability to bind to an intracellular target protein.

5 The present invention encompasses antisense nucleic acid molecules, i.e., molecules which are complementary to a sense nucleic acid encoding a protein, e.g., complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. Accordingly, an antisense nucleic acid can hydrogen bond to a sense nucleic acid. The antisense nucleic acid can be complementary to an entire CARD-12 coding strand, or to only a portion thereof, e.g., all or part of the protein coding region (or open reading frame). An antisense nucleic acid molecule can be antisense to a noncoding region of the coding strand of a nucleotide sequence encoding CARD-12. The noncoding regions ("5' and 3' untranslated regions") are the 5' and 3' sequences that flank the coding region and are not translated into amino acids.

10 Given the coding strand sequences encoding CARD-12 disclosed herein, antisense nucleic acids of the invention can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule can be complementary to the entire coding region of CARD-12 mRNA, but more preferably is an oligonucleotide which is antisense to only a portion of the coding or noncoding region of CARD-12 mRNA. For example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of CARD-12 mRNA. An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length. An antisense nucleic acid of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (e.g., an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, e.g., phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil,

beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v),
 5 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (i.e., RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest, described further in the following
 10 subsection).

The antisense nucleic acid molecules of the invention are typically administered to a subject or generated in situ such that they hybridize with or bind to cellular mRNA and/or genomic DNA encoding a CARD-12 protein to thereby inhibit expression of the protein, e.g., by inhibiting transcription and/or translation. The hybridization can be by
 15 conventional nucleotide complementarity to form a stable duplex, or, for example, in the case of an antisense nucleic acid molecule which binds to DNA duplexes, through specific interactions in the major groove of the double helix. An antisense nucleic acid molecule of the invention can be administered by direct injection at a tissue site. Alternatively, antisense nucleic acid molecules can be modified to target selected cells and then administered systemically. For example, for systemic administration, antisense
 20 molecules can be modified such that they specifically bind to receptors or antigens expressed on a selected cell surface, e.g., by linking the antisense nucleic acid molecules to peptides or antibodies which bind to cell surface receptors or antigens. The antisense nucleic acid molecules can also be delivered to cells using the vectors described herein.
 25 To achieve sufficient intracellular concentrations of the antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong pol II or pol III promoter are preferred.

An antisense nucleic acid molecule of the invention can be an α -anomeric nucleic acid molecule. An α -anomeric nucleic acid molecule forms specific double-stranded
 30 hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gaultier et al. (1987) Nucleic Acids. Res. 15:6625-6641). The antisense nucleic acid molecule can also comprise a 2'-o-methylribonucleotide (Inoue et al. (1987) Nucleic Acids Res. 15:6131-6148) or a chimeric RNA-DNA analogue (Inoue et al. (1987) FEBS Lett. 215:327-330).

35 The invention also encompasses ribozymes. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded

nucleic acid, such as an mRNA, to which they have a complementary region. Thus, ribozymes (e.g., hammerhead ribozymes (described in Haselhoff and Gerlach (1988) Nature 334:585-591)) can be used to catalytically cleave CARD-12 mRNA transcripts to thereby inhibit translation of CARD-12 mRNA. A ribozyme having specificity for a
5 CARD-12-encoding nucleic acid can be designed based upon the nucleotide sequence of a CARD-12 cDNA disclosed herein. For example, a derivative of a Tetrahymena L-19 IVS RNA can be constructed in which the nucleotide sequence of the active site is complementary to the nucleotide sequence to be cleaved in a CARD-12-encoding mRNA. See, e.g., Cech et al. U.S. Patent No. 4,987,071; and Cech et al. U.S. Patent No.
10 5,116,742. Alternatively, CARD-12 mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See, e.g., Bartel and Szostak (1993) Science 261:1411-1418.

The invention also encompasses nucleic acid molecules which form triple helical structures. For example, CARD-12 gene expression can be inhibited by targeting
15 nucleotide sequences complementary to the regulatory region of the CARD-12 (e.g., the CARD-12 promoter and/or enhancers) to form triple helical structures that prevent transcription of the CARD-12 gene in target cells. See generally, Helene (1991) Anticancer Drug Des. 6(6):569-84; Helene (1992) Ann. N.Y. Acad. Sci. 660:27-36; and Maher (1992) Bioassays 14(12):807-15.

In embodiments, the nucleic acid molecules of the invention can be modified at the base moiety, sugar moiety or phosphate backbone to improve, e.g., the stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate backbone of the nucleic acids can be modified to generate peptide nucleic acids (see
20 Hyrup et al. (1996) Bioorganic & Medicinal Chemistry 4(1):5-23). As used herein, the terms "peptide nucleic acids" or "PNAs" refer to nucleic acid mimics, e.g., DNA mimics, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral backbone of PNAs has been shown to allow for specific hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid
25 phase peptide synthesis protocols as described in Hyrup et al. (1996) supra; Perry-O'Keefe et al. (1996) Proc. Natl. Acad. Sci. USA 93:14670-675.

PNAs of CARD-12 can be used for therapeutic and diagnostic applications. For example, PNAs can be used as antisense or antigene agents for sequence-specific modulation of gene expression by, e.g., inducing transcription or translation arrest or
30 inhibiting replication. PNAs of CARD-12 can also be used, e.g., in the analysis of single base pair mutations in a gene by, e.g., PNA directed PCR clamping; as artificial

restriction enzymes when used in combination with other enzymes, e.g., S1 nucleases (Hyrup (1996) supra; or as probes or primers for DNA sequence and hybridization (Hyrup (1996) supra; Perry-O'Keefe et al. (1996) Proc. Natl. Acad. Sci. USA 93: 14670-675).

5 In another embodiment, PNAs of CARD-12 can be modified, e.g., to enhance their stability or cellular uptake, by attaching lipophilic or other helper groups to PNA, by the formation of PNA-DNA chimeras, or by the use of liposomes or other techniques of drug delivery known in the art. For example, PNA-DNA chimeras of CARD-12 can be generated which may combine the advantageous properties of PNA and DNA. Such
10 chimeras allow DNA recognition enzymes, e.g., RNase H and DNA polymerases, to interact with the DNA portion while the PNA portion would provide high binding affinity and specificity. PNA-DNA chimeras can be linked using linkers of appropriate lengths selected in terms of base stacking, number of bonds between the nucleobases, and orientation (Hyrup (1996) supra). The synthesis of PNA-DNA chimeras can be
15 performed as described in Hyrup (1996) supra and Finn et al. (1996) Nucleic Acids Research 24(17):3357-63. For example, a DNA chain can be synthesized on a solid support using standard phosphoramidite coupling chemistry and modified nucleoside analogs, e.g., 5'-(4-methoxytrityl)amino-5'-deoxy-thymidine phosphoramidite, can be used as a between the PNA and the 5' end of DNA (Mag et al. (1989) Nucleic Acid Res.
20 17:5973-88). PNA monomers are then coupled in a stepwise manner to produce a chimeric molecule with a 5' PNA segment and a 3' DNA segment (Finn et al. (1996) Nucleic Acids Research 24(17):3357-63). Alternatively, chimeric molecules can be synthesized with a 5' DNA segment and a 3' PNA segment (Peterser et al. (1975) Bioorganic Med. Chem. Lett. 5:1119-11124).

25 In other embodiments, the oligonucleotide may include other appended groups such as peptides (e.g., for targeting host cell receptors in vivo), or agents facilitating transport across the cell membrane (see, e.g., Letsinger et al. (1989) Proc. Natl. Acad. Sci. USA 86:6553-6556; Lemaitre et al. (1987) Proc. Natl. Acad. Sci. USA 84:648-652; PCT Publication No. W0 88/09810) or the blood-brain barrier (see, e.g., PCT Publication
30 No. W0 89/10134). In addition, oligonucleotides can be modified with hybridization-triggered cleavage agents (see, e.g., Krol et al. (1988) Bio/Techniques 6:958-976) or intercalating agents (see, e.g., Zon (1988) Pharm. Res. 5:539-549). To this end, the oligonucleotide may be conjugated to another molecule, e.g., a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered
35 cleavage agent, etc.

II. Isolated CARD-12 Proteins and Anti-CARD-12 Antibodies.

One aspect of the invention pertains to isolated CARD-12 proteins, and biologically active portions thereof, as well as polypeptide fragments suitable for use as immunogens to raise anti-CARD-12 antibodies. In one embodiment, native CARD-12 proteins can be isolated from cells or tissue sources by an appropriate purification scheme using standard protein purification techniques. In another embodiment, CARD-12 proteins are produced by recombinant DNA techniques. Alternative to recombinant expression, a CARD-12 protein or polypeptide can be synthesized chemically using standard peptide synthesis techniques.

An "isolated" or "purified" protein or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the CARD-12 protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of CARD-12 protein in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. Thus, CARD-12 protein that is substantially free of cellular material includes preparations of CARD-12 protein having less than about 30%, 20%, 10%, or 5% (by dry weight) of non-CARD-12 protein (also referred to herein as a "contaminating protein"). When the CARD-12 protein or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, i.e., culture medium represents less than about 20%, 10%, or 5% of the volume of the protein preparation. When CARD-12 protein is produced by chemical synthesis, it is preferably substantially free of chemical precursors or other chemicals, i.e., it is separated from chemical precursors or other chemicals which are involved in the synthesis of the protein. Accordingly such preparations of CARD-12 protein have less than about 30%, 20%, 10%, 5% (by dry weight) of chemical precursors or non-CARD-12 chemicals.

Biologically active portions of a CARD-12 protein include peptides comprising amino acid sequences sufficiently identical to or derived from the amino acid sequence of the CARD-12 protein (e.g., the amino acid sequence shown in SEQ ID NO:2), which include less amino acids than the full length CARD-12 protein, and exhibit at least one activity of a CARD-12 protein. Typically, biologically active portions comprise a domain or motif with at least one activity of the CARD-12 protein. A biologically active portion of a CARD-12 protein can be a polypeptide which is, for example, 10, 25, 50, 100, 150, 200, 250, 300 or more amino acids in length. Preferred biologically active

polypeptides include one or more identified CARD-12 structural domains, e.g., the CARD domain (amino acids 1-88 of SEQ ID NO:2).

Moreover, other biologically active portions, in which other regions of the protein are deleted, can be prepared by recombinant techniques and evaluated for one or more of the functional activities of a native CARD-12 protein.

CARD-12 protein has the amino acid sequence shown of SEQ ID NO:2. Other useful CARD-12 proteins are substantially identical to SEQ ID NO:2 and retain the functional activity of the protein of SEQ ID NO:2, yet differ in amino acid sequence due to natural allelic variation or mutagenesis.

A useful CARD-12 protein is a protein which includes an amino acid sequence at least about 45%, preferably 55%, 65%, 75%, 85%, 95%, or 99% identical to the amino acid sequence of SEQ ID NO:2, and retains the functional activity of the CARD-12 protein of SEQ ID NO:2.

To determine the percent identity of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = # of identical positions/total # of positions x 100).

The determination of percent homology between two sequences can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul (1990) Proc. Nat'l Acad. Sci. USA 87:2264-2268, modified as in Karlin and Altschul (1993) Proc. Nat'l Acad. Sci. USA 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, et al. (1990) J. Mol. Biol. 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences similar or homologous to CARD-12 nucleic acid molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al. (1997) Nucleic Acids Res. 25:3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See <http://www.ncbi.nlm.nih.gov>. Another

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1911 1912 1913 1914 1915 1916 1917 1918 1919 1920 1921 1922 1923 1924 1925 1926 1927 1928 1929 1930 1931 1932 1933 1934 1935 1936 1937 1938 1939 1940 1941 1942 1943 1944 1945 1946 1947 1948 1949 1950 1951 1952 1953 1954 1955 1956 1957 1958 1959 1960 1961 1962 1963 1964 1965 1966 1967 1968 1969 1970 1971 1972 1973 1974 1975 1976 1977 1978 1979 1980 1981 1982 1983 1984 1985 1986 1987 1988 1989 1990 1991 1992 1993 1994 1995 1996 1997 1998 1999 2000 2001 2002 2003 2004 2005 2006 2007 2008 2009 2010 2011 2012 2013 2014 2015 2016 2017 2018 2019 2020 2021 2022 2023 2024 2025 2026 2027 2028 2029 2030 2031 2032 2033 2034 2035 2036 2037 2038 2039 2040 2041 2042 2043 2044 2045 2046 2047 2048 2049 2050 2051 2052 2053 2054 2055 2056 2057 2058 2059 2060 2061 2062 2063 2064 2065 2066 2067 2068 2069 2070 2071 2072 2073 2074 2075 2076 2077 2078 2079 2080 2081 2082 2083 2084 2085 2086 2087 2088 2089 2090 2091 2092 2093 2094 2095 2096 2097 2098 2099 2100 2101 2102 2103 2104 2105 2106 2107 2108 2109 2110 2111 2112 2113 2114 2115 2116 2117 2118 2119 2120 2121 2122 2123 2124 2125 2126 2127 2128 2129 2130 2131 2132 2133 2134 2135 2136 2137 2138 2139 2140 2141 2142 2143 2144 2145 2146 2147 2148 2149 2150 2151 2152 2153 2154 2155 2156 2157 2158 2159 2160 2161 2162 2163 2164 2165 2166 2167 2168 2169 2170 2171 2172 2173 2174 2175 2176 2177 2178 2179 2180 2181 2182 2183 2184 2185 2186 2187 2188 2189 2190 2191 2192 2193 2194 2195 2196 2197 2198 2199 2200 2201 2202 2203 2204 2205 2206 2207 2208 2209 2210 2211 2212 2213 2214 2215 2216 2217 2218 2219 2220 2221 2222 2223 2224 2225 2226 2227 2228 2229 2230 2231 2232 2233 2234 2235 2236 2237 2238 2239 2240 2241 2242 2243 2244 2245 2246 2247 2248 2249 2250 2251 2252 2253 2254 2255 2256 2257 2258 2259 2260 2261 2262 2263 2264 2265 2266 2267 2268 2269 2270 2271 2272 2273 2274 2275 2276 2277 2278 2279 2280 2281 2282 2283 2284 2285 2286 2287 2288 2289 2290 2291 2292 2293 2294 2295 2296 2297 2298 2299 2300 2301 2302 2303 2304 2305 2306 2307 2308 2309 2310 2311 2312 2313 2314 2315 2316 2317 2318 2319 2320 2321 2322 2323 2324 2325 2326 2327 2328 2329 2330 2331 2332 2333 2334 2335 2336 2337 2338 2339 2340 2341 2342 2343 2344 2345 2346 2347 2348 2349 2350 2351 2352 2353 2354 2355 2356 2357 2358 2359 2360 2361 2362 2363 2364 2365 2366 2367 2368 2369 2370 2371 2372 2373 2374 2375 2376 2377 2378 2379 2380 2381 2382 2383 2384 2385 2386 2387 2388 2389 2390 2391 2392 2393 2394 2395 2396 2397 2398 2399 2400 2401 2402 2403 2404 2405 2406 2407 2408 2409 2410 2411 2412 2413 2414 2415 2416 2417 2418 2419 2420 2421 2422 2423 2424 2425 2426 2427 2428 2429 2430 2431 2432 2433 2434 2435 2436 2437 2438 2439 2440 2441 2442 2443 2444 2445 2446 2447 2448 2449 2450 2451 2452 2453 2454 2455 2456 2457 2458 2459 2460 2461 2462 2463 2464 2465 2466 2467 2468 2469 2470 2471 2472 2473 2474 2475 2476 2477 2478 2479 2480 2481 2482 2483 2484 2485 2486 2487 2488 2489 2490 2491 2492 2493 2494 2495 2496 2497 2498 2499 2500 2501 2502 2503 2504 2505 2506 2507 2508 2509 2510 2511 2512 2513 2514 2515 2516 2517 2518 2519 2520 2521 2522 2523 2524 2525 2526 2527 2528 2529 2530 2531 2532 2533 2534 2535 2536 2537 2538 2539 2540 2541 2542 2543 2544 2545 2546 2547 2548 2549 2550 2551 2552 2553 2554 2555 2556 2557 2558 2559 2560 2561 2562 2563 2564 2565 2566 2567 2568 2569 2570 2571 2572 2573 2574 2575 2576 2577 2578 2579 2580 2581 2582 2583 2584 2585 2586 2587 2588 2589 2590 2591 2592 2593 2594 2595 2596 2597 2598 2599 2600 2601 2602 2603 2604 2605 2606 2607 2608 2609 2610 2611 2612 2613 2614 2615 2616 2617 2618 2619 2620 2621 2622 2623 2624 2625 2626 2627 2628 2629 2630 2631 2632 2633 2634 2635 2636 2637 2638 2639 2640 2641 2642 2643 2644 2645 2646 2647 2648 2649 2650 2651 2652 2653 2654 2655 2656 2657 2658 2659 2660 2661 2662 2663 2664 2665 2666 2667 2668 2669 2670 2671 2672 2673 2674 2675 2676 2677 2678 2679 2680 2681 2682 2683 2684 2685 2686 2687 2688 2689 2690 2691 2692 2693 2694 2695 2696 2697 2698 2699 2700 2701 2702 2703 2704 2705 2706 2707 2708 2709 2710 2711 2712 2713 2714 2715 2716 2717 2718 2719 2720 2721 2722 2723 2724 2725 2726 2727 2728 2

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more of the activities of the naturally occurring form of the CARD-12 protein by, for example, competitively binding to a downstream or upstream member of a cellular signaling cascade which includes the CARD-12 protein. Thus, specific biological effects can be elicited by treatment with a variant of limited function. Treatment of a subject
 5 with a variant having a subset of the biological activities of the naturally occurring form of the protein can have fewer side effects in a subject relative to treatment with the naturally occurring form of the CARD-12 proteins.

Variants of the CARD-12 protein which function as either CARD-12 agonists (mimetics) or as CARD-12 antagonists can be identified by screening combinatorial
 10 libraries of mutants, e.g., truncation mutants of the CARD-12 protein for CARD-12 protein agonist or antagonist activity. In one embodiment, a variegated library of CARD-12 variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A variegated library of CARD-12 variants can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides
 15 into gene sequences such that a degenerate set of potential CARD-12 sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g., for phage display) containing the set of CARD-12 sequences therein. There are a variety of methods which can be used to produce libraries of potential CARD-12 variants from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene
 20 sequence can be performed in an automatic DNA synthesizer, and the synthetic gene then ligated into an appropriate expression vector. Use of a degenerate set of genes allows for the provision, in one mixture, of all of the sequences encoding the desired set of potential CARD-12 sequences. Methods for synthesizing degenerate oligonucleotides are known in the art (see, e.g., Narang (1983) Tetrahedron 39:3; Itakura et al. (1984) Annu. Rev.
 25 Biochem. 53:323; Itakura et al. (1984) Science 198:1056; Ike et al. (1983) Nucleic Acid Res. 11:477).

Useful fragments of CARD-12, include fragments comprising or consisting of a domain or subdomain described herein, e.g., a kinase-2 domain or a CARD domain.

In addition, libraries of fragments of the CARD-12 protein coding sequence can
 30 be used to generate a variegated population of CARD-12 fragments for screening and subsequent selection of variants of a CARD-12 protein. In one embodiment, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of a CARD-12 coding sequence with a nuclease under conditions wherein nicking occurs only about once per molecule, denaturing the double stranded DNA, renaturing the DNA
 35 to form double stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment

with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes N-terminal and internal fragments of various sizes of the CARD-12 protein.

Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property. Such techniques are adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of CARD-12 proteins. The most widely used techniques, which are amenable to high through-put analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recursive ensemble mutagenesis (REM), a technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify CARD-12 variants (Arkin and Yourvan (1992) *Proc. Natl. Acad. Sci. USA* 89:7811-7815; Delgrave et al. (1993) *Protein Engineering* 6(3):327-331).

An isolated CARD-12 protein, or a portion or fragment thereof, can be used as an immunogen to generate antibodies that bind CARD-12 using standard techniques for polyclonal and monoclonal antibody preparation. The full-length CARD-12 protein can be used or, alternatively, the invention provides antigenic peptide fragments of CARD-12 for use as immunogens. The antigenic peptide of CARD-12 comprises at least 8 (preferably 10, 15, 20, or 30) amino acid residues of the amino acid sequence shown in SEQ ID NO:2 and encompasses an epitope of CARD-12 such that an antibody raised against the peptide forms a specific immune complex with CARD-12.

Useful antibodies include antibodies which bind to a domain or subdomain of CARD-12 described herein (e.g., a kinase-2 domain, a CARD domain, an NBS domain, a P-loop domain, or a LRR domain).

Preferred epitopes encompassed by the antigenic peptide are regions of CARD-12 that are located on the surface of the protein, e.g., hydrophilic regions. Other important criteria include a preference for a terminal sequence, high antigenic index (e.g., as predicted by Jameson-Wolf algorithm), ease of peptide synthesis (e.g., avoidance of prolines); and high surface probability (e.g., as predicted by the Emini algorithm; Figure 4).

A CARD-12 immunogen typically is used to prepare antibodies by immunizing a suitable subject, (e.g., rabbit, goat, mouse or other mammal) with the immunogen. An

appropriate immunogenic preparation can contain, for example, recombinantly expressed CARD-12 protein or a chemically synthesized CARD-12 polypeptide. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Immunization of a suitable subject with an immunogenic CARD-12 preparation induces a polyclonal anti-CARD-12 antibody response.

Accordingly, another aspect of the invention pertains to anti-CARD-12 antibodies. The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site which specifically binds an antigen, such as CARD-12. A molecule which specifically binds to CARD-12 is a molecule which binds CARD-12, but does not substantially bind other molecules in a sample, e.g., a biological sample, which naturally contains CARD-12. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and F(ab')₂ fragments which can be generated by treating the antibody with an enzyme such as pepsin. The invention provides polyclonal and monoclonal antibodies that bind CARD-12. The term "monoclonal antibody" or "monoclonal antibody composition", as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of CARD-12. A monoclonal antibody composition thus typically displays a single binding affinity for a particular CARD-12 protein with which it immunoreacts.

Polyclonal anti-CARD-12 antibodies can be prepared as described above by immunizing a suitable subject with a CARD-12 immunogen. The anti-CARD-12 antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized CARD-12. If desired, the antibody molecules directed against CARD-12 can be isolated from the mammal (e.g., from the blood) and further purified by well-known techniques, such as protein A chromatography to obtain the IgG fraction. At an appropriate time after immunization, e.g., when the anti-CARD-12 antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975) *Nature* 256:495-497, the human B cell hybridoma technique (Kozbor et al. (1983) *Immunol Today* 4:72), the EBV-hybridoma technique (Cole et al. (1985), *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96) or trioma techniques. The technology for producing various antibodies monoclonal antibody hybridomas is well known (see

generally Current Protocols in Immunology (1994) Coligan et al. (eds.) John Wiley & Sons, Inc., New York, NY). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with a CARD-12 immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds CARD-12.

Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti-CARD-12 monoclonal antibody (see, e.g., Current Protocols in Immunology, supra; Galfre et al. (1977) Nature 266:55052; R.H. Kenneth, in Monoclonal Antibodies: A New Dimension In Biological Analyses, Plenum Publishing Corp., New York, New York (1980); and Lerner (1981) Yale J. Biol. Med., 54:387-402). Moreover, the ordinarily skilled worker will appreciate that there are many variations of such methods which also would be useful. Typically, the immortal cell line (e.g., a myeloma cell line) is derived from the same mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the present invention with an immortalized mouse cell line, e.g., a myeloma cell line that is sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines can be used as a fusion partner according to standard techniques, e.g., the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from ATCC. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody of the invention are detected by screening the hybridoma culture supernatants for antibodies that bind CARD-12, e.g., using a standard ELISA assay.

Alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal anti-CARD-12 antibody can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (e.g., an antibody phage display library) with CARD-12 to thereby isolate immunoglobulin library members that bind CARD-12. Kits for generating and screening phage display libraries are commercially available (e.g., the Pharmacia Recombinant Phage Antibody System, Catalog No. 27-9400-01; and the Stratagene SurfZAP Phage Display Kit, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in

generating and screening antibody display library can be found in, for example, U.S. Patent No. 5,223,409; PCT Publication No. WO 92/18619; PCT Publication No. WO 91/17271; PCT Publication No. WO 92/20791; PCT Publication No. WO 92/15679; PCT Publication No. WO 93/01288; PCT Publication No. WO 92/01047; PCT Publication No. WO 92/09690; PCT Publication No. WO 90/02809; Fuchs et al. (1991) *Bio/Technology* 9:1370-1372; Hay et al. (1992) *Hum. Antibod. Hybridomas* 3:81-85; Huse et al. (1989) *Science* 246:1275-1281; Griffiths et al. (1993) *EMBO J.* 12:725-734.

Additionally, recombinant anti-CARD-12 antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in PCT Publication No. WO 87/02671; European Patent Application 184,187; European Patent Application 171,496; European Patent Application 173,494; PCT Publication No. WO 86/01533; U.S. Patent No. 4,816,567; European Patent Application 125,023; Better et al. (1988) *Science* 240:1041-1043; Liu et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:3439-3443; Liu et al. (1987) *J. Immunol.* 139:3521-3526; Sun et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:214-218; Nishimura et al. (1987) *Canc. Res.* 47:999-1005; Wood et al. (1985) *Nature* 314:446-449; and Shaw et al. (1988) *J. Natl. Cancer Inst.* 80:1553-1559; Morrison, (1985) *Science* 229:1202-1207; Oi et al. (1986) *Bio/Techniques* 4:214; U.S. Patent 5,225,539; Jones et al. (1986) *Nature* 321:552-525; Verhoeyan et al. (1988) *Science* 239:1534; and Beidler et al. (1988) *J. Immunol.* 141:4053-4060.

An anti-CARD-12 antibody (e.g., monoclonal antibody) can be used to isolate CARD-12 by standard techniques, such as affinity chromatography or immunoprecipitation. An anti-CARD-12 antibody can facilitate the purification of natural CARD-12 from cells and of recombinantly produced CARD-12 expressed in host cells. Moreover, an anti-CARD-12 antibody can be used to detect CARD-12 protein (e.g., in a cellular lysate or cell supernatant) in order to evaluate the abundance and pattern of expression of the CARD-12 protein. Anti-CARD-12 antibodies can be used diagnostically to monitor protein levels in tissue as part of a clinical testing procedure, e.g., to, for example, determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β -galactosidase,

or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include ^{125}I , ^{131}I , ^{35}S or ^3H .

Further, an antibody (or fragment thereof) may be conjugated to a therapeutic moiety such as a cytotoxin, a therapeutic agent or a radioactive metal ion. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine).

The conjugates of the invention can be used for modifying a given biological response. The drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor, α -interferon, β -interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator; or, biological response modifiers such as, for example, lymphokines, interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6), granulocyte macrophase colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), or other growth factors.

Techniques for conjugating such therapeutic moiety to antibodies are well known, see, e.g., Arnon et al., "Monoclonal Antibodies for Immunotargeting of Drugs in Cancer Therapy", in Monoclonal Antibodies and Cancer Therapy, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom et al., "Antibodies for Drug Delivery", in

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the formulation instonic with the blood of the recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents or thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampoules and vials and may be stored in a freeze-dried condition requiring only the addition of the sterile liquid carrier immediately prior to use. The vaccine formulation may also include adjuvant systems for enhancing the immunogenicity of the formulation, such as oil-in water systems and other systems known in the art. The dosage will depend on the specific activity of the vaccine and can be readily determined by routine experimentation.

III. Computer Readable Means

The nucleotide or amino acid sequences of the invention are also provided in a variety of mediums to facilitate use thereof. As used herein, "provided" refers to a manufacture, other than an isolated nucleic acid or amino acid molecule, which contains a nucleotide or amino acid sequence of the present invention. Such a manufacture provides the nucleotide or amino acid sequences, or a subset thereof (e.g., a subset of open reading frames (ORFs)) in a form which allows a skilled artisan to examine the manufacture using means not directly applicable to examining the nucleotide or amino acid sequences, or a subset thereof, as they exist in nature or in purified form.

In one application of this embodiment, a nucleotide or amino acid sequence of the present invention can be recorded on computer readable media. As used herein, "computer readable media" refers to any medium that can be read and accessed directly by a computer. Such media include, but are not limited to: magnetic storage media, such as floppy discs, hard disc storage medium, and magnetic tape; optical storage media such as CD-ROM; electrical storage media such as RAM and ROM; and hybrids of these categories such as magnetic/optical storage media. This skilled artisan will readily appreciate how any of the presently known computer readable mediums can be used to create a manufacture comprising computer readable medium having recorded thereon a nucleotide or amino acid sequence of the present invention.

As used herein, "recorded" refers to a process for storing information on computer readable medium. The skilled artisan can readily adopt any of the presently known methods for recording information on computer readable medium to generate manufactures comprising the nucleotide or amino acid sequence information of the present invention.

A variety of data storage structures are available to a skilled artisan for creating a computer readable medium having recorded thereon a nucleotide or amino acid sequence

of the present invention. The choice of the data storage structure will generally be based on the means chosen to access the stored information. In addition, a variety of data processor programs and formats can be used to store the nucleotide sequence information of the present invention on computer readable medium. The sequence information can be represented in a work processing test file, formatted in commercially-available software such as WordPerfect and Microsoft Word, or represented in the form of an ASCII file, stored in a database application, such as DB2, Sybase, Oracle, or the like. The skilled artisan can readily adapt any number of data processor structuring formats (e.g., text file or database) in order to obtain computer readable medium having recorded thereon the nucleotide sequence information of the present invention.

By providing the nucleotide or amino acid sequences of the invention in computer readable form, the skilled artisan can routinely access the sequence information for a variety of purposes. For example, one skilled in the art can use the nucleotide or amino acid sequences of the invention in computer readable form to compare a target sequence or a target structural motif with the sequence information stored within the data storage means. Search means are used to identify fragments or regions of the sequences of the invention which match a particular target sequence or target motif.

As used herein, a "target sequence" can be any DNA or amino acid sequence of six or more nucleotides or two or more amino acids. A skilled artisan can readily recognize that the longer a target sequence is, the less likely a target sequence will be present as a random occurrence in the database. The most preferred sequence length of a target sequence is from about 10 to 100 amino acids or from about 30 to 300 nucleotide residues. However, it is well recognized that commercially important fragments, such as sequence fragments involved in gene expression and protein processing, may be of shorter length.

As used herein, "a target structural motif," or "target motif," refers to any rationally selected sequence or combination of sequences in which the sequence(s) are chosen based on a three-dimensional configuration formed upon the folding of the target motif. There are a variety of target motifs known in the art. Protein target motifs include, but are not limited to, enzyme active sites and signal sequences. Nucleic acid target motifs include, but are not limited to, promoter sequences, hairpin structures and inducible expression elements (protein binding sequences).

Computer software is publicly available which allows a skilled artisan to access sequence information provided in a computer readable medium for analysis and comparison to other sequences. A variety of known algorithms are disclosed publicly and a variety of commercially available software for conducting search means are and can be

used in the computer-based systems of the present invention. Examples of such software include, but is not limited to, MacPattern (EMBL), BLASTIN and BLASTX (NCBIA).

For example, software that implements the BLAST (*Altschul et al. (1990) J. of Mol. Biol. 215:403-410*) and BLAZE (*Brutlag et al. (1993) Comp. Chem. 17:203-207*) search algorithms on a Sybase system can be used to identify open reading frames (ORFs) of the sequences of the invention which contain homology to ORFs or proteins from other libraries. Such ORFs are protein-encoding fragments and are useful in producing commercially important proteins such as enzymes used in various reactions and in the production of commercially useful metabolites.

IV. Recombinant Expression Vectors and Host Cells

Another aspect of the invention pertains to vectors, preferably expression vectors, containing a nucleic acid encoding CARD-12 (or a portion thereof). As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors, expression vectors, are capable of directing the expression of genes to which they are operatively linked. In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids (vectors). However, the invention is intended to include such other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The recombinant expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (e.g., in an in vitro transcription/translation system or in a host cell

when the vector is introduced into the host cell). The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (e.g., polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cell and those which direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (e.g., CARD-12 proteins, mutant forms of CARD-12, fusion proteins, etc.).

The recombinant expression vectors of the invention can be designed for expression of CARD-12 in prokaryotic or eukaryotic cells, e.g., bacterial cells such as E. coli, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). Alternatively, the recombinant expression vector can be transcribed and translated in vitro, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in E. coli with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith and Johnson (1988) Gene 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amann et al., (1988) *Gene* 69:301-315) and pET 11d (Studier et al., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 60-89). Target gene expression from the pTrc vector relies on host RNA
 5 polymerase transcription from a hybrid trp-lac fusion promoter. Target gene expression from the pET 11d vector relies on transcription from a T7 gn10-lac fusion promoter mediated by a coexpressed viral RNA polymerase (T7 gn1). This viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident λ prophage harboring a T7 gn1 gene under the transcriptional control of the lacUV5 promoter.

10 One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a bacterial having an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 119-128). Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so
 15 that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada et al. (1992) *Nucleic Acids Res.* 20:2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques.

In another embodiment, the CARD-12 expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1
 20 (Baldari et al. (1987) *EMBO J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz et al. (1987) *Gene* 54:113-123), pYES2 (Invitrogen Corporation, San Diego, CA), pGBT9 (Clontech, Palo Alto, CA), pGAD10 (Clontech, Palo Alto, CA), pYADE4 and pYGAE2 and pYPGE2 (Brunelli and Pall, (1993) *Yeast* 9:1299-1308), pYPGE15 (Brunelli and Pall, (1993) *Yeast* 9:1309-1318), pACTII (Dr.
 25 S.E. Elledge, Baylor College of Medicine), and picZ (Invitrogen Corp, San Diego, CA).

Alternatively, CARD-12 can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (e.g., Sf 9 cells) include the pAc series (Smith et al. (1983) *Mol. Cell Biol.* 3:2156-2165) and the pVL series (Lucklow and Summers (1989) *Virology* 170:31-39).

30 In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed (1987) *Nature* 329:840), pCI (Promega), and pMT2PC (Kaufman et al. (1987) *EMBO J.* 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements.

35 For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both

prokaryotic and eukaryotic cells see chapters 16 and 17 of Sambrook et al. (supra).

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (e.g., tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert et al. (1987) *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji et al. (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (e.g., the neurofilament promoter; Byrne and Ruddle (1989) *Proc. Natl. Acad. Sci. USA* 86:5473-5477), pancreas-specific promoters (Edlund et al. (1985) *Science* 230:912-916), and mammary gland-specific promoters (e.g., milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166).

Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the α -fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546).

The invention further provides a recombinant expression vector comprising a DNA molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner which allows for expression (by transcription of the DNA molecule) of an RNA molecule which is antisense to CARD-12 mRNA. Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the continuous expression of the antisense RNA molecule in a variety of cell types, for instance viral promoters and/or enhancers, or regulatory sequences can be chosen which direct constitutive, tissue specific or cell type specific expression of antisense RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub et al. (Reviews - Trends in Genetics, Vol. 1(1) 1986).

Another aspect of the invention pertains to host cells into which a recombinant expression vector of the invention or isolated nucleic acid molecule of the invention has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular

subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

5 A host cell can be any prokaryotic or eukaryotic cell. For example, CARD-12 protein can be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art.

10 Vector DNA or an isolated nucleic acid molecule of the invention can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (e.g., DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. 15 Suitable methods for transforming or transfecting host cells can be found in Sambrook, et al. (*supra*), and other laboratory manuals.

20 For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In some cases vector DNA is retained by the host cell. In other cases the host cell does not retain vector DNA and retains only an isolated nucleic acid molecule of the invention carried by the vector. In some cases, and isolated nucleic acid molecule of the invention is used to transform a cell without the use of a vector.

25 In order to identify and select these integrants, a gene that encodes a selectable marker (e.g., resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding CARD-12 or can be introduced on a separate vector. Cells stably transfected with the 30 introduced nucleic acid can be identified by drug selection (e.g., cells that have incorporated the selectable marker gene will survive, while the other cells die).

35 A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (i.e., express) a CARD-12 protein. Accordingly, the invention further provides methods for producing CARD-12 protein using the host cells of the invention. In one embodiment, the method comprises culturing the host cell of the invention (into which a recombinant expression vector or isolated nucleic acid molecule

encoding CARD-12 has been introduced) in a suitable medium such that CARD-12 protein is produced. In another embodiment, the method further comprises isolating CARD-12 from the medium or the host cell.

5 CARD-12 nucleic acid molecules can be used in viral gene delivery systems for gene therapy, e.g., adenoviral or retroviral gene delivery systems.

CARD-12 nucleic acid molecules can also be used in non-viral gene delivery systems for gene therapy. Thus, another aspect of the invention pertains to non-viral gene delivery systems, such as plasmid-based gene delivery systems. Non-viral gene delivery systems are described in detail by Huang et al. ((1999) Nonviral Vectors for
10 Gene Therapy, Academic Press, San Diego, CA). Nonviral vectors have several potential advantages over their viral counterparts, including: reduced immunogenicity; low acute toxicity; simplicity; and ease of large scale production. Nonviral vectors can be delivered as naked DNA, by bioballistic bombardment, and in various complexes, including liposome/DNA complexes (lipoplexes), polymer/DNA complexes (polyplexes), and
15 liposome/polymer/DNA complexes (lipopolyplexes). Nonviral vectors may be administered by various routes, e.g., intravenous injection, peritoneal injection, intramuscular injection, subcutaneous injection, intratracheal injection, and aerosolization.

Naked DNA (i.e. free from association with, e.g., transfection-facilitating
20 proteins, viral particles, liposomal formulations, charged lipids and calcium phosphate precipitating), can be expressed at its injection site or at a remote site. For example, naked DNA can be injected directly into skeletal muscle, liver, heart muscle, and tumor tissue. For systemic administration, plasmid DNA may need to be protected from degradation by endonucleases during delivery from the site of administration to the site
25 of gene expression.

Bioballistic bombardment, also known as gene gun, allows for the penetration of target cells *in vitro*, *ex vivo*, or *in vivo*. In this technique, DNA-coated gold particles are accelerated to a high velocity by an electric arc generated by a high voltage discharge. The method is effective for a variety of organ types, including skin, liver, muscle, spleen,
30 and pancreas. The gene gun transfer method is not dependent upon specific cell surface receptors, cell cycle status, or the size of the DNA vector. Useful gene gun devices include the Accell® (PowderJect Vaccines, Inc.) and the Helios™ (Bio-Rad). These devices create a compressed shock wave of helium gas, accelerating DNA-coated gold (or tungsten) particles to high speed, whereby the particles have sufficient momentum to
35 penetrate a target tissue.

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altered. Such animals are useful for studying the function and/or activity of CARD-12 and for identifying and/or evaluating modulators of CARD-12 activity. As used herein, a "transgenic animal" is a non-human animal, preferably a mammal, more preferably a rodent such as a rat or mouse, in which one or more of the cells of the animal includes a transgene. Other examples of transgenic animals include non-human primates, sheep, dogs, cows, goats, chickens, amphibians, etc. A transgene is exogenous DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, thereby directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal. As used herein, an "homologous recombinant animal" is a non-human animal, preferably a mammal, more preferably a mouse, in which an endogenous CARD-12 gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, e.g., an embryonic cell of the animal, prior to development of the animal.

A transgenic animal of the invention can be created by introducing CARD-12-encoding nucleic acid into the male pronuclei of a fertilized oocyte, e.g., by microinjection, retroviral infection, and allowing the oocyte to develop in a pseudopregnant female foster animal. The CARD-12 cDNA sequence, e.g., that of SEQ ID NO:1, SEQ ID NO:3 or the cDNA of ATCC _____ can be introduced as a transgene into the genome of a non-human animal. Alternatively, a nonhuman homolog or ortholog of the human CARD-12 gene, such as a mouse CARD-12 gene, can be isolated based on hybridization to the human CARD-12 cDNA and used as a transgene. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. A tissue-specific regulatory sequence(s) can be operably linked to the CARD-12 transgene to direct expression of CARD-12 protein to particular cells. Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009, U.S. Patent No. 4,873,191 and in Hogan, Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other transgenic animals. A transgenic founder animal can be identified based upon the presence of the CARD-12 transgene in its genome and/or expression of CARD-12 mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying a transgene encoding CARD-12 can further be bred to other transgenic animals carrying other transgenes.

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Acad. Sci. USA 89:6232-6236. Another example of a recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman et al. (1991) Science 251:1351-1355. If a cre/loxP recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the Cre recombinase and a selected protein are required. Such animals can be provided through the construction of "double" transgenic animals, e.g., by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase.

Clones of the non-human transgenic animals described herein can also be produced according to the methods described in Wilmut et al. (1997) Nature 385:810-813 and PCT Publication Nos. WO 97/07668 and WO 97/07669. In brief, a cell, e.g., a somatic cell, from the transgenic animal can be isolated and induced to exit the growth cycle and enter Go phase. The quiescent cell can then be fused, e.g., through the use of electrical pulses, to an enucleated oocyte from an animal of the same species from which the quiescent cell is isolated. The reconstructed oocyte is then cultured such that it develops to morula or blastocyte and then transferred to pseudopregnant female foster animal. The offspring borne of this female foster animal will be a clone of the animal from which the cell, e.g., the somatic cell, is isolated.

V. Pharmaceutical Compositions

The CARD-12 nucleic acid molecules, CARD-12 proteins, and anti-CARD-12 antibodies (also referred to herein as "active compounds") of the invention can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the nucleic acid molecule, protein, or antibody and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

The invention includes methods for preparing pharmaceutical compositions for modulating the expression or activity of a polypeptide or nucleic acid of the invention. Such methods comprise formulating a pharmaceutically acceptable carrier with an agent which modulates expression or activity of a polypeptide or nucleic acid of the invention.

Such compositions can further include additional active agents. Thus, the invention further includes methods for preparing a pharmaceutical composition by formulating a pharmaceutically acceptable carrier with an agent which modulates expression or activity of a polypeptide or nucleic acid of the invention and one or more additional active compounds.

The agent which modulates expression or activity may, for example, be a small molecule. For example, such small molecules include peptides, peptidomimetics, amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, organic or inorganic compounds (i.e., including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds. It is understood that appropriate doses of small molecule agents depends upon a number of factors within the ken of the ordinarily skilled physician, veterinarian, or researcher. The dose(s) of the small molecule will vary, for example, depending upon the identity, size, and condition of the subject or sample being treated, further depending upon the route by which the composition is to be administered, if applicable, and the effect which the practitioner desires the small molecule to have upon the nucleic acid or polypeptide of the invention. Exemplary doses include milligram or microgram amounts of the small molecule per kilogram of subject or sample weight (e.g., about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram. It is furthermore understood that appropriate doses of a small molecule depend upon the potency of the small molecule with respect to the expression or activity to be modulated. Such appropriate doses may be determined using the assays described herein. When one or more of these small molecules is to be administered to an animal (e.g., a human) in order to modulate expression or activity of a polypeptide or nucleic acid of the invention, a physician, veterinarian, or researcher may, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an appropriate response is obtained. In addition, it is understood that the specific dose level for any particular subject will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, gender, and diet of the subject, the time

of administration, the route of administration, the rate of excretion, any drug combination, and the degree of expression or activity to be modulated.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL (BASF; Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (e.g., a CARD-12 protein or anti-CARD-12 antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring. For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

For antibodies, the preferred dosage is 0.1 mg/kg to 100 mg/kg of body weight (generally 10 mg/kg to 20 mg/kg). If the antibody is to act in the brain, a dosage of 50 mg/kg to 100 mg/kg is usually appropriate. Generally, partially human antibodies and fully human antibodies have a longer half-life within the human body than other antibodies. Accordingly, lower dosages and less frequent administration is often possible. Modifications such as lipidation can be used to stabilize antibodies and to enhance uptake and tissue penetration (e.g., into the brain). A method for lipidation of antibodies is described by Cruikshank et al. ((1997) J. Acquired Immune Deficiency Syndromes and Human Retrovirology 14:193).

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (U.S. Patent 5,328,470) or by stereotactic injection (see, e.g., Chen et al. (1994) Proc. Natl. Acad. Sci. USA

91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

The gene therapy vectors of the invention can be either viral or non-viral. Examples of plasmid-based, non-viral vectors are discussed in Huang et al. (1999) Nonviral Vectors for Gene Therapy (supra). A modified plasmid is one example of a non-viral gene delivery system. Peptides, proteins (including antibodies), and oligonucleotides may be stably conjugated to plasmid DNA by methods that do not interfere with the transcriptional activity of the plasmid (Zelphati et al. (2000) BioTechniques 28:304-315). The attachment of proteins and/or oligonucleotides may influence the delivery and trafficking of the plasmid and thus render it a more effective pharmaceutical composition.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

VI. Uses and Methods of the Invention

The nucleic acid molecules, proteins, protein homologues, and antibodies described herein can be used in one or more of the following methods: a) screening assays; b) detection assays (e.g., chromosomal mapping, tissue typing, forensic biology), c) predictive medicine (e.g., diagnostic assays, prognostic assays, monitoring clinical trials, and pharmacogenomics); and d) methods of treatment (e.g., therapeutic and prophylactic). A CARD-12 protein interacts with other cellular proteins and can thus be used for (i) regulation of cellular proliferation; (ii) regulation of cellular differentiation; and (iii) regulation of cell survival. The isolated nucleic acid molecules of the invention can be used to express CARD-12 protein (e.g., via a recombinant expression vector in a host cell in gene therapy applications), to detect CARD-12 mRNA (e.g., in a biological sample) or a genetic lesion in a CARD-12 gene, and to modulate CARD-12 activity. In addition, the CARD-12 proteins can be used to screen drugs or compounds which modulate the CARD-12 activity or expression as well as to treat disorders characterized by insufficient or excessive production of CARD-12 protein or production of CARD-12 protein forms which have decreased or aberrant activity compared to CARD-12 wild type protein. In addition, the anti-CARD-12 antibodies of the invention can be used to detect and isolate CARD-12 proteins and modulate CARD-12 activity.

This invention further pertains to novel agents identified by the above-described screening assays and uses thereof for treatments as described herein.

A. Screening Assays

The invention provides a method (also referred to herein as a "screening assay") for identifying modulators, i.e., candidate or test compounds or agents (e.g., peptides, peptidomimetics, small molecules or other drugs) which bind to CARD-12 proteins or biologically active portions thereof or have a stimulatory or inhibitory effect on, for example, CARD-12 expression or CARD-12 activity. An example of a biologically active portion of human CARD-12 is amino acids 139-227 encoding a CARD domain.

Among the screening assays provided by the invention are screening to identify molecules that prevent the dimerization of CARD-12, screening to identify molecules which block the binding of a CARD containing polypeptide to CARD-12, and screening to identify a competitive inhibitor of the binding of a nucleotide to the nucleotide binding site of CARD-12. Screening assays, e.g., dimerization assays, can employ full-length CARD-12 or a portion of CARD-12, e.g, the CARD domain, the nucleotide binding site domain, or the NAIP homology domain.

In one embodiment, the invention provides assays for screening candidate or test compounds which bind to or modulate the activity of a CARD-12 proteins or polypeptides or biologically active portions thereof. The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the "one-bead one-compound" library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam (1997) *Anticancer Drug Des.* 12:145). Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt et al. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909; Erb et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann et al. (1994). *J. Med. Chem.* 37:2678; Cho et al. (1993) *Science* 261:1303; Carrell et al. (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell et al. (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and Gallop et al. (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds may be presented in solution (e.g., Houghten (1992) *Bio/Techniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (U.S. Patent No. 5,223,409), spores (Patent Nos.

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the ability of the test compound to preferentially bind to CARD-12 or biologically active portion thereof as compared to the known binding compound.

In another embodiment, an assay is a cell-free assay comprising contacting CARD-12 protein or biologically active portion thereof with a test compound and
 5 determining the ability of the test compound to modulate (e.g., stimulate or inhibit) the activity of the CARD-12 protein or biologically active portion thereof. Determining the ability of the test compound to modulate the activity of CARD-12 can be accomplished, for example, by determining the ability of the CARD-12 protein to bind to a CARD-12 target molecule by one of the methods described above for determining direct binding. In
 10 an alternative embodiment, determining the ability of the test compound to modulate the activity of CARD-12 can be accomplished by determining the ability of the CARD-12 protein to further modulate a CARD-12 target molecule. For example, the catalytic/enzymatic activity of the target molecule on an appropriate substrate can be determined as previously described.

In yet another embodiment, the cell-free assay comprises contacting the CARD-12 protein or biologically active portion thereof with a known compound which binds CARD-12 to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with a CARD-12 protein, wherein determining the ability of the test compound to interact with a CARD-12 protein
 15 comprises determining the ability of the CARD-12 protein to preferentially bind to or modulate the activity of a CARD-12 target molecule. The cell-free assays of the present invention are amenable to use of either the soluble form or a membrane-associated form of CARD-12. A membrane-associated form of CARD-12 refers to CARD-12 that interacts with a membrane-bound target molecule. In the case of cell-free assays
 20 comprising the membrane-associated form of CARD-12, it may be desirable to utilize a solubilizing agent such that the membrane-associated form of CARD-12 is maintained in solution. Examples of such solubilizing agents include non-ionic detergents such as n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-N-methylglucamide, decanoyl-N-methylglucamide, Triton® X-100, Triton® X-114, Thesit®,
 25 Isotridecylpoly(ethylene glycol ether)n,
 30 3-[(3-cholamidopropyl)dimethylamminio]-1-propane sulfonate (CHAPS),
 3-[(3-cholamidopropyl)dimethylamminio]-2-hydroxy-1-propane sulfonate (CHAPSO), or N-dodecyl=N,N-dimethyl-3-ammonio-1-propane sulfonate.

In more than one embodiment of the above assay methods of the present
 35 invention, it may be desirable to immobilize either CARD-12 or its target molecule to facilitate separation of complexed from uncomplexed forms of one or both of the

proteins, as well as to accommodate automation of the assay. Binding of a test compound to CARD-12, or interaction of CARD-12 with a target molecule in the presence and absence of a candidate compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows one or both of the proteins to be bound to a matrix. For example, glutathione-S-transferase/CARD-12 fusion proteins or glutathione-S-transferase/target fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical; St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the test compound or the test compound and either the non-adsorbed target protein or CARD-12 protein, and the mixture incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Following incubation, the beads or microtitre plate wells are washed to remove any unbound components, the matrix immobilized in the case of beads, complex determined either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the matrix, and the level of CARD-12 binding or activity determined using standard techniques. In an alternative embodiment, MYC or HA epitope tag CARD-12 fusion proteins or MYC or HA epitope tag target fusion proteins can be adsorbed onto anti-MYC or anti-HA antibody coated microbeads or onto anti-MYC or anti-HA antibody coated microtitre plates, which are then combined with the test compound or the test compound and either the non-adsorbed target protein or CARD-12 protein, and the mixture incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Following incubation, the beads or microtitre plate wells are washed to remove any unbound components, the matrix immobilized in the case of beads, complex determined either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the matrix, and the level of CARD-12 binding or activity determined using standard techniques.

Other techniques for immobilizing proteins on matrices can also be used in the screening assays of the invention. For example, CARD-12 or its target molecule can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated CARD-12 target molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals; Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with CARD-12 or target molecules but which do not interfere with binding of the protein to its target molecule can be derivatized to the wells

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assay utilizes two different DNA constructs. In one construct, the gene that codes for CARD-12 is fused to a gene encoding the DNA binding domain of a known transcription factor (e.g., GAL-4). In the other construct, a DNA sequence, from a library of DNA sequences, that encodes an unidentified protein ("prey" or "sample") is fused to a gene that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact, in vivo, forming a CARD-12-dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (e.g., LacZ) which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected and cell colonies containing the functional transcription factor can be isolated and used to obtain the cloned gene which encodes the protein which interacts with CARD-12.

In an embodiment of the invention, the ability of a test compound to modulate the activity of CARD-12, or a biologically active portion thereof can be determined by assaying the ability of the test compound to block the binding of CARD-12 to its target proteins in a yeast or mammalian two-hybrid system assay. This assay could be automated for high throughput drug screening purposes. In another embodiment of the invention, CARD-12 and a target protein could be configured in the reverse two-hybrid system (Vidal et al. (1996) Proc. Natl. Acad. Sci. USA 93:10321-6 and Vidal et al. (1996) Proc. Natl. Acad. Sci. USA 93:10315-20) designed specifically for efficient drug screening. In the reverse two-hybrid system, inhibition of a CARD-12 physical interaction with a target protein would result in induction of a reporter gene in contrast to the normal two-hybrid system where inhibition of CARD-12 physical interaction with a target protein would lead to reporter gene repression. The reverse two-hybrid system is preferred for drug screening because reporter gene induction is more easily assayed than report gene repression.

Alternative embodiments of the invention are proteins found to physically interact with proteins that bind to CARD-12. CARD-12 interactors could be configured into two-hybrid system baits and used in two-hybrid screens to identify additional members of the CARD-12 pathway. The interactors of CARD-12 interactors identified in this way could be useful targets for therapeutic intervention in CARD-12 related diseases and pathologies and an assay of their enzymatic or binding activity could be useful for the identification of test compounds that modulate CARD-12 activity.

This invention further pertains to novel agents identified by the above-described screening assays and uses thereof for treatments as described herein.

B. Detection Assays

Portions or fragments of the cDNA sequences identified herein (and the corresponding complete gene sequences) can be used in numerous ways as polynucleotide reagents. For example, these sequences can be used to: (i) map their
 5 respective genes on a chromosome; and, thus, locate gene regions associated with genetic disease; (ii) identify an individual from a minute biological sample (tissue typing); and (iii) aid in forensic identification of a biological sample. These applications are described in the subsections below.

1. Chromosome Mapping

10 Once the sequence (or a portion of the sequence) of a gene has been isolated, this sequence can be used to map the location of the gene on a chromosome. Accordingly, CARD-12 nucleic acid molecules described herein or fragments thereof, can be used to map the location of CARD-12 genes on a chromosome. The mapping of the CARD-12 sequences to chromosomes is an important first step in correlating these sequences with
 15 genes associated with disease.

Briefly, CARD-12 genes can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp in length) from the CARD-12 sequences. Computer analysis of CARD-12 sequences can be used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process.
 20 These primers can then be used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the CARD-12 sequences will yield an amplified fragment.

Somatic cell hybrids are prepared by fusing somatic cells from different mammals (e.g., human and mouse cells). As hybrids of human and mouse cells grow and divide,
 25 they gradually lose human chromosomes in random order, but retain the mouse chromosomes. By using media in which mouse cells cannot grow, because they lack a particular enzyme, but human cells can, the one human chromosome that contains the gene encoding the needed enzyme, will be retained. By using various media, panels of hybrid cell lines can be established. Each cell line in a panel contains either a single
 30 human chromosome or a small number of human chromosomes, and a full set of mouse chromosomes, allowing easy mapping of individual genes to specific human chromosomes. (D'Eustachio et al. (1983) Science 220:919-924). Somatic cell hybrids containing only fragments of human chromosomes can also be produced using human chromosomes with translocations and deletions.

35 PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular sequence to a particular chromosome. Three or more sequences can be

assigned per day using a single thermal cycler. Using the CARD-12 sequences to design oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes. Other mapping strategies which can similarly be used to map a CARD-12 sequence to its chromosome include in situ hybridization (described in Fan et al. (1990) Proc. Natl. Acad. Sci. USA 87:6223-27), pre-screening with labeled flow-sorted chromosomes, and pre-selection by hybridization to chromosome specific cDNA libraries.

Fluorescence in situ hybridization (FISH) of a DNA sequence to a metaphase chromosomal spread can further be used to provide a precise chromosomal location in one step. Chromosome spreads can be made using cells whose division has been blocked in metaphase by a chemical like colcemid that disrupts the mitotic spindle. The chromosomes can be treated briefly with trypsin, and then stained with Giemsa. A pattern of light and dark bands develops on each chromosome, so that the chromosomes can be identified individually. The FISH technique can be used with a DNA sequence as short as 500 or 600 bases. However, clones larger than 1,000 bases have a higher likelihood of binding to a unique chromosomal location with sufficient signal intensity for simple detection. Preferably 1,000 bases, and more preferably 2,000 bases will suffice to get good results at a reasonable amount of time. For a review of this technique, see Verma et al., (Human Chromosomes: A Manual of Basic Techniques (Pergamon Press, New York, 1988)).

Reagents for chromosome mapping can be used individually to mark a single chromosome or a single site on that chromosome, or panels of reagents can be used for marking multiple sites and/or multiple chromosomes. Reagents corresponding to noncoding regions of the genes actually are preferred for mapping purposes. Coding sequences are more likely to be conserved within gene families, thus increasing the chance of cross hybridizations during chromosomal mapping.

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. (Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man, available on-line through Johns Hopkins University Welch Medical Library). The relationship between genes and disease, mapped to the same chromosomal region, can then be identified through linkage analysis (co-inheritance of physically adjacent genes), described in, e.g., Egeland et al. (1987) Nature, 325:783-787.

Moreover, differences in the DNA sequences between individuals affected and unaffected with a disease associated with the CARD-12 gene can be determined. If a mutation is observed in some or all of the affected individuals but not in any unaffected

individuals, then the mutation is likely to be the causative agent of the particular disease. Comparison of affected and unaffected individuals generally involves first looking for structural alterations in the chromosomes such as deletions or translocations that are visible from chromosome spreads or detectable using PCR based on that DNA sequence.

5 Ultimately, complete sequencing of genes from several individuals can be performed to confirm the presence of a mutation and to distinguish mutations from polymorphisms.

A CARD-12 polypeptide and fragments and sequences thereof and antibodies specific thereto can be used to map the location of the gene encoding the polypeptide on a chromosome. This mapping can be carried out by specifically detecting the presence of
 10 the polypeptide in members of a panel of somatic cell hybrids between cells of a first species of animal from which the protein originates and cells from a second species of animal and then determining which somatic cell hybrid(s) expresses the polypeptide and noting the chromosome(s) from the first species of animal that it contains. For examples of this technique, see Pajunen *et al.* (1988) *Cytogenet. Cell Genet.* 47:37-41 and Van
 15 Keuren *et al.* (1986) *Hum. Genet.* 74:34-40. Alternatively, the presence of the CARD-12 polypeptide in the somatic cell hybrids can be determined by assaying an activity or property of the polypeptide, for example, enzymatic activity, as described in Bordelon-Riser *et al.* (1979) *Somatic Cell Genetics* 5:597-613 and Owerbach *et al.* (1978) *Proc. Natl. Acad. Sci. USA* 75:5640-5644.

20 2. Tissue Typing

The CARD-12 sequences of the present invention can also be used to identify individuals from minute biological samples. The United States military, for example, is considering the use of restriction fragment length polymorphism (RFLP) for
 25 identification of its personnel. In this technique, an individual's genomic DNA is digested with one or more restriction enzymes, and probed on a Southern blot to yield unique bands for identification. This method does not suffer from the current limitations of "Dog Tags" which can be lost, switched, or stolen, making positive identification difficult. The sequences of the present invention are useful as additional DNA markers for RFLP (described in U.S. Patent 5,272,057).

30 Furthermore, the sequences of the present invention can be used to provide an alternative technique which determines the actual base-by-base DNA sequence of selected portions of an individual's genome. Thus, the CARD-12 sequences described herein can be used to prepare two PCR primers from the 5' and 3' ends of the sequences. These primers can then be used to amplify an individual's DNA and subsequently
 35 sequence it.

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generated fragments. Sequences targeted to noncoding regions of SEQ ID NO:1 are particularly appropriate for this use as greater numbers of polymorphisms occur in the noncoding regions, making it easier to differentiate individuals using this technique. Examples of polynucleotide reagents include the CARD-12 sequences or portions thereof, e.g., fragments derived from the noncoding regions of SEQ ID NO:1 which have a length of at least 20 or 30 bases.

The sequences described herein can further be used to provide polynucleotide reagents, e.g., labeled or labelable probes which can be used in, for example, an in situ hybridization technique, to identify a specific tissue, e.g., brain tissue. This can be very useful in cases where a forensic pathologist is presented with a tissue of unknown origin. Panels of such CARD-12 probes can be used to identify tissue by species and/or by organ type.

In a similar fashion, these reagents, e.g., CARD-12 primers or probes can be used to screen tissue culture for contamination (i.e., screen for the presence of a mixture of different types of cells in a culture).

C. Predictive Medicine

The present invention also pertains to the field of predictive medicine in which diagnostic assays, prognostic assays, pharmacogenomics, and monitoring clinical trials are used for prognostic (predictive) purposes to thereby treat an individual prophylactically. Accordingly, one aspect of the present invention relates to diagnostic assays for determining CARD-12 protein and/or nucleic acid expression as well as CARD-12 activity, in the context of a biological sample (e.g., blood, serum, cells, tissue) to thereby determine whether an individual is afflicted with a disease or disorder, or is at risk of developing a disorder, associated with aberrant CARD-12 expression or activity. The invention also provides for prognostic (or predictive) assays for determining whether an individual is at risk of developing a disorder associated with CARD-12 protein, nucleic acid expression or activity. For example, mutations in a CARD-12 gene can be assayed in a biological sample. Such assays can be used for prognostic or predictive purpose to thereby prophylactically treat an individual prior to the onset of a disorder characterized by or associated with CARD-12 protein, nucleic acid expression or activity.

Another aspect of the invention provides methods for determining CARD-12 protein, nucleic acid expression or CARD-12 activity in an individual to thereby select appropriate therapeutic or prophylactic agents for that individual (referred to herein as "pharmacogenomics"). Pharmacogenomics allows for the selection of agents (e.g., drugs) for therapeutic or prophylactic treatment of an individual based on the genotype of

the individual (e.g., the genotype of the individual examined to determine the ability of the individual to respond to a particular agent.)

Yet another aspect of the invention pertains to monitoring the influence of agents (e.g., drugs or other compounds) on the expression or activity of CARD-12 in clinical trials.

These and other agents are described in further detail in the following sections.

1. Diagnostic Assays

An exemplary method for detecting the presence or absence of CARD-12 in a biological sample involves obtaining a biological sample from a test subject and contacting the biological sample with a compound or an agent capable of detecting CARD-12 protein or nucleic acid (e.g., mRNA, genomic DNA) that encodes CARD-12 protein such that the presence of CARD-12 is detected in the biological sample. An agent for detecting CARD-12 mRNA or genomic DNA is a labeled nucleic acid probe capable of hybridizing to CARD-12 mRNA or genomic DNA. The nucleic acid probe can be, for example, a full-length CARD-12 nucleic acid, such as the nucleic acid of SEQ ID NO:1 or 3, or a portion thereof, such as an oligonucleotide of at least 15, 30, 50, 100, 250 or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to mRNA or genomic DNA. Other suitable probes for use in the diagnostic assays of the invention are described herein.

An agent for detecting CARD-12 protein can be an antibody capable of binding to CARD-12 protein, preferably an antibody with a detectable label. Antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof (e.g., Fab or F(ab')₂) can be used. The term "labeled", with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (i.e., physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin. The term "biological sample" is intended to include tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject. That is, the detection method of the invention can be used to detect CARD-12 mRNA, protein, or genomic DNA in a biological sample in vitro as well as in vivo. For example, in vitro techniques for detection of CARD-12 mRNA include Northern hybridizations and in situ hybridizations. In vitro techniques for detection of CARD-12 protein include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and

immunofluorescence. In vitro techniques for detection of CARD-12 genomic DNA include Southern hybridizations. Furthermore, in vivo techniques for detection of CARD-12 protein include introducing into a subject a labeled anti-CARD-12 antibody. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques.

In one embodiment, the biological sample contains protein molecules from the test subject. Alternatively, the biological sample can contain mRNA molecules from the test subject or genomic DNA molecules from the test subject. A biological sample is a peripheral blood leukocyte sample isolated by conventional means from a subject.

In another embodiment, the methods further involve obtaining a control biological sample from a control subject, contacting the control sample with a compound or agent capable of detecting CARD-12 protein, mRNA, or genomic DNA, such that the presence of CARD-12 protein, mRNA or genomic DNA is detected in the biological sample, and comparing the presence of CARD-12 protein, mRNA or genomic DNA in the control sample with the presence of CARD-12 protein, mRNA or genomic DNA in the test sample.

The invention also encompasses kits for detecting the presence of CARD-12 in a biological sample (a test sample). Such kits can be used to determine if a subject is suffering from or is at increased risk of developing a disorder associated with aberrant expression of CARD-12 (e.g., an immunological disorder). For example, the kit can comprise a labeled compound or agent capable of detecting CARD-12 protein or mRNA in a biological sample and means for determining the amount of CARD-12 in the sample (e.g., an anti-CARD-12 antibody or an oligonucleotide probe which binds to DNA encoding CARD-12, e.g., SEQ ID NO:1 or SEQ ID NO:3). Kits may also include instruction for observing that the tested subject is suffering from or is at risk of developing a disorder associated with aberrant expression of CARD-12 if the amount of CARD-12 protein or mRNA is above or below a normal level.

For antibody-based kits, the kit may comprise, for example: (1) a first antibody (e.g., attached to a solid support) which binds to CARD-12 protein; and, optionally, (2) a second, different antibody which binds to CARD-12 protein or the first antibody and is conjugated to a detectable agent. For oligonucleotide-based kits, the kit may comprise, for example: (1) an oligonucleotide, e.g., a detectably labelled oligonucleotide, which hybridizes to a CARD-12 nucleic acid sequence or (2) a pair of primers useful for amplifying a CARD-12 nucleic acid molecule.

The kit may also comprise, e.g., a buffering agent, a preservative, or a protein stabilizing agent. The kit may also comprise components necessary for detecting the

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techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

In an alternative embodiment, mutations in a CARD-12 gene from a sample cell can be identified by alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, amplified (optionally), digested with one or more restriction endonucleases, and fragment length sizes are determined by gel electrophoresis and compared. Differences in fragment length sizes between sample and control DNA indicates mutations in the sample DNA. Moreover, the use of sequence specific ribozymes (see, e.g., U.S. Patent No. 5,498,531) can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage site.

In other embodiments, genetic mutations in CARD-12 can be identified by hybridizing a sample and control nucleic acids, e.g., DNA or RNA, to high density arrays containing hundreds or thousands of oligonucleotides probes (Cronin et al. (1996) Human Mutation 7:244-255; Kozal et al. (1996) Nature Medicine 2:753-759). For example, genetic mutations in CARD-12 can be identified in two-dimensional arrays containing light-generated DNA probes as described in Cronin et al. supra. Briefly, a first hybridization array of probes can be used to scan through long stretches of DNA in a sample and control to identify base changes between the sequences by making linear arrays of sequential overlapping probes. This step allows the identification of point mutations. This step is followed by a second hybridization array that allows the characterization of specific mutations by using smaller, specialized probe arrays complementary to all variants or mutations detected. Each mutation array is composed of parallel probe sets, one complementary to the wild-type gene and the other complementary to the mutant gene.

In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence the CARD-12 gene and detect mutations by comparing the sequence of the sample CARD-12 with the corresponding wild-type (control) sequence. Examples of sequencing reactions include those based on techniques developed by Maxam and Gilbert ((1977) Proc. Natl. Acad. Sci. USA 74:560) or Sanger ((1977) Proc. Natl. Acad. Sci. USA 74:5463). It is also contemplated that any of a variety of automated sequencing procedures can be utilized when performing the diagnostic assays ((1995) Bio/Techniques 19:448), including sequencing by mass spectrometry (see, e.g., PCT Publication No. WO 94/16101; Cohen et al. (1996) Adv. Chromatogr. 36:127-162; and Griffin et al. (1993) Appl. Biochem. Biotechnol. 38:147-159).

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1. The first group of people who are interested in the results of the study are the researchers themselves. They want to know how well the study was conducted and whether the results are reliable and valid. They also want to know how the study can be used to inform future research.

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the presence of a known mutation at a specific site by looking for the presence or absence of amplification.

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one probe nucleic acid or antibody reagent described herein, which may be conveniently used, e.g., in clinical settings to diagnose patients exhibiting symptoms or family history of a disease or illness involving a CARD-12 gene.

Furthermore, any cell type or tissue, preferably peripheral blood leukocytes, in which CARD-12 is expressed may be utilized in the prognostic assays described herein.

3. Pharmacogenomics

Agents, or modulators which have a stimulatory or inhibitory effect on CARD-12 activity (e.g., CARD-12 gene expression) as identified by a screening assay described herein can be administered to individuals to treat (prophylactically or therapeutically) disorders (e.g., an immunological disorder) associated with aberrant CARD-12 activity. In conjunction with such treatment, the pharmacogenomics (i.e., the study of the relationship between an individual's genotype and that individual's response to a foreign compound or drug) of the individual may be considered. Differences in metabolism of therapeutics can lead to severe toxicity or therapeutic failure by altering the relation between dose and blood concentration of the pharmacologically active drug. Thus, the pharmacogenomics of the individual permits the selection of effective agents (e.g., drugs) for prophylactic or therapeutic treatments based on a consideration of the individual's genotype. Such pharmacogenomics can further be used to determine appropriate dosages and therapeutic regimens. Accordingly, the activity of CARD-12 protein, expression of CARD-12 nucleic acid, or mutation content of CARD-12 genes in an individual can be determined to thereby select appropriate agent(s) for therapeutic or prophylactic treatment of the individual.

Pharmacogenomics deals with clinically significant hereditary variations in the response to drugs due to altered drug disposition and abnormal action in affected persons. See, e.g., Linder (1997) Clin. Chem. 43(2):254-266. In general, two types of pharmacogenetic conditions can be differentiated. Genetic conditions transmitted as a single factor altering the way drugs act on the body (altered drug action) or genetic conditions transmitted as single factors altering the way the body acts on drugs (altered drug metabolism). These pharmacogenetic conditions can occur either as rare defects or as polymorphisms. For example, glucose-6-phosphate dehydrogenase deficiency (G6PD) is a common inherited enzymopathy in which the main clinical complication is

haemolysis after ingestion of oxidant drugs (anti-malarials, sulfonamides, analgesics, nitrofurans) and consumption of fava beans.

As an illustrative embodiment, the activity of drug metabolizing enzymes is a major determinant of both the intensity and duration of drug action. The discovery of genetic polymorphisms of drug metabolizing enzymes (e.g., N-acetyltransferase 2 (NAT 2) and cytochrome P450 enzymes CYP2D6 and CYP2C19) has provided an explanation as to why some patients do not obtain the expected drug effects or show exaggerated drug response and serious toxicity after taking the standard and safe dose of a drug. These polymorphisms are expressed in two phenotypes in the population, the extensive metabolizer (EM) and poor metabolizer (PM). The prevalence of PM is different among different populations. For example, the gene coding for CYP2D6 is highly polymorphic and several mutations have been identified in PM, which all lead to the absence of functional CYP2D6. Poor metabolizers of CYP2D6 and CYP2C19 quite frequently experience exaggerated drug response and side effects when they receive standard doses. If a metabolite is the active therapeutic moiety, PM exhibit no therapeutic response, as demonstrated for the analgesic effect of codeine mediated by its CYP2D6-formed metabolite morphine. The other extreme are the so-called ultra-rapid metabolizers who do not respond to standard doses. Recently, the molecular basis of ultra-rapid metabolism has been identified to be due to CYP2D6 gene amplification.

Thus, the activity of CARD-12 protein, expression of CARD-12 nucleic acid, or mutation content of CARD-12 genes in an individual can be determined to thereby select appropriate agent(s) for therapeutic or prophylactic treatment of the individual. In addition, pharmacogenetic studies can be used to apply genotyping of polymorphic alleles encoding drug-metabolizing enzymes to the identification of an individual's drug responsiveness phenotype. This knowledge, when applied to dosing or drug selection, can avoid adverse reactions or therapeutic failure and thus enhance therapeutic or prophylactic efficiency when treating a subject with a CARD-12 modulator, such as a modulator identified by one of the exemplary screening assays described herein.

4. Monitoring of Effects During Clinical Trials

Monitoring the influence of agents (e.g., drugs, compounds) on the expression or activity of CARD-12 (e.g., the ability to modulate aberrant cell proliferation and/or differentiation) can be applied not only in basic drug screening, but also in clinical trials. For example, the effectiveness of an agent determined by a screening assay as described herein to increase CARD-12 gene expression, protein levels, or upregulate CARD-12 activity, can be monitored in clinical trials of subjects exhibiting decreased CARD-12 gene expression, protein levels, or downregulated CARD-12 activity. Alternatively, the

effectiveness of an agent determined by a screening assay to decrease CARD-12 gene expression, protein levels, or downregulated CARD-12 activity, can be monitored in clinical trials of subjects exhibiting increased CARD-12 gene expression, protein levels, or upregulated CARD-12 activity. In such clinical trials, the expression or activity of

5 CARD-12 and, preferably, other genes that have been implicated in, for example, a cellular proliferation disorder can be used as a "read out" or markers of the immune responsiveness of a particular cell.

For example, and not by way of limitation, genes, including CARD-12, that are modulated in cells by treatment with an agent (e.g., compound, drug or small molecule)

10 which modulates CARD-12 activity (e.g., identified in a screening assay as described herein) can be identified. Thus, to study the effect of agents on cellular proliferation disorders, for example, in a clinical trial, cells can be isolated and RNA prepared and analyzed for the levels of expression of CARD-12 and other genes implicated in the disorder. The levels of gene expression (i.e., a gene expression pattern) can be quantified

15 by Northern blot analysis or RT-PCR, as described herein, or alternatively by measuring the amount of protein produced, by one of the methods as described herein, or by measuring the levels of activity of CARD-12 or other genes. In this way, the gene expression pattern can serve as a marker, indicative of the physiological response of the cells to the agent. Accordingly, this response state may be determined before, and at

20 various points during, treatment of the individual with the agent.

In an embodiment, the present invention provides a method for monitoring the effectiveness of treatment of a subject with an agent (e.g., an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate identified by the screening assays described herein) comprising the steps of (i) obtaining

25 a pre-administration sample from a subject prior to administration of the agent; (ii) detecting the level of expression of a CARD-12 protein, mRNA, or genomic DNA in the preadministration sample; (iii) obtaining one or more post-administration samples from the subject; (iv) detecting the level of expression or activity of the CARD-12 protein, mRNA, or genomic DNA in the post-administration samples; (v) comparing the level of

30 expression or activity of the CARD-12 protein, mRNA, or genomic DNA in the pre-administration sample with the CARD-12 protein, mRNA, or genomic DNA in the post administration sample or samples; and (vi) altering the administration of the agent to the subject accordingly. For example, increased administration of the agent may be desirable to increase the expression or activity of CARD-12 to higher levels than

35 detected, i.e., to increase the effectiveness of the agent. Alternatively, decreased

administration of the agent may be desirable to decrease expression or activity of CARD-12 to lower levels than detected, i.e., to decrease the effectiveness of the agent.

5. Transcriptional Profiling

The CARD-12 nucleic acid molecules described herein, including small oligonucleotides, can be used in transcriptionally profiling. For example, these nucleic acids can be used to examine the expression of CARD-12 in normal tissue or cells and in tissue or cells subject to a disease state, e.g., tissue or cells derived from a patient having a disease of interest or cultured cells which model or reflect a disease state of interest, e.g., cells of a cultured tumor cell line. By measuring expression of CARD-12, together or individually, a profile of expression in normal and disease states can be developed. This profile can be used diagnostically and to examine the effectiveness of a therapeutic regime.

C. Methods of Treatment

The present invention provides for both prophylactic and therapeutic methods of treating a subject at risk of (or susceptible to) a disorder or having a disorder associated with aberrant CARD-12 expression or activity, examples of which are provided herein.

1. Prophylactic Methods

In one aspect, the invention provides a method for preventing in a subject, a disease or condition associated with an aberrant CARD-12 expression or activity, by administering to the subject an agent which modulates CARD-12 expression or at least one CARD-12 activity. Subjects at risk for a disease which is caused or contributed to by aberrant CARD-12 expression or activity can be identified by, for example, any or a combination of diagnostic or prognostic assays as described herein. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of the CARD-12 aberrancy, such that a disease or disorder is prevented or, alternatively, delayed in its progression. Depending on the type of CARD-12 aberrancy, for example, a CARD-12 agonist or CARD-12 antagonist agent can be used for treating the subject. The appropriate agent can be determined based on screening assays described herein.

2. Therapeutic Methods

Another aspect of the invention pertains to methods of modulating CARD-12 expression or activity for therapeutic purposes. The modulatory method of the invention involves contacting a cell with an agent that modulates one or more of the activities of CARD-12 protein activity associated with the cell. An agent that modulates CARD-12 protein activity can be an agent as described herein, such as a nucleic acid or a protein, a naturally-occurring cognate ligand of a CARD-12 protein, a peptide, a CARD-12

1. The first part of the report is a general introduction to the subject of the study. It discusses the importance of the study and the objectives of the research.

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1. **Introduction**
 2. **Background**
 3. **Methodology**
 4. **Results**
 5. **Conclusion**
 6. **References**

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